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*TISSUE STORAGE OF MUCOPOLYSACCHARIDES IN HÜRLER-PFAUNDLER'S DISEASE**

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Conflicting reports have been published on the nature of the storage substance which accumulates in the spleen, liver, and other tissues of patients with Hürler-Pfaundler's disease ("gargoylism"). Brante[1] reported that a sulfated mucopolysaccharide of seemingly simple constitution was present in large amounts in one autopsy liver specimen. Uzman[2] found two principal components—one a polysaccharide fraction having hexoses, hexosamines, and sulfate and the other a glycolipid of complex composition. Stacey[3] has reported the presence of sulfated polysaccharide fractions as well as of nonsulfated fractions, the latter somewhat related to blood group-specific substances.

Isolation of Storage Substance.—In the present work we have examined tissues removed at autopsy from (*a*) one normal child dying from an acute respiratory tract infection, (*b*) and (*c*) two fatal cases of Hürler-Pfaundler's disease in children, and (*d*) liver tissue removed by biopsy from the liver of a child with this disease. It has been found that homogenization of liver or spleen in 10 volumes of cold 5 per cent trichloroacetic acid (TCA) in a Waring Blendor extracts at least 80 per cent of the storage substance, as well as any glycogen which is present. A study of the TCA-insoluble residue has shown that it contains not over 20 per cent additional material which is related in structure to the soluble storage substance described below. Following removal of TCA by ethyl ether extraction, the storage substance and the glycogen are precipitated completely by 2 volumes of ethanol in the presence of 1 per cent potassium acetate and 1 per cent calcium chloride (pH 5.7). In the case of tissue from a gargoyle patient, the voluminous precipitate is mucoid in character. An aqueous solution of the precipitate is centrifuged at 100,000 × *g* for 2 hours to sediment a large part of any glycogen present. None of the characteristic storage substance is present in the pellet. An additional amount of glycogen can be removed from the supernatant solution by enzymatic digestion with phosphorylase and amylo-1,6-glucosidase, followed by ethanol precipitation of the mucopolysaccharide which remains. This latter material has been subjected to one or more of the following procedures: alcohol fractionation in the presence of calcium acetate; direct column chromatography on Dowex-1

(formate); continuous paper electrophoresis-chromatography. The properties of some of these fractions are shown in Table 1.

TABLE 1

PROPERTIES OF SOME MUCOPOLYSACCHARIDE FRACTIONS FROM HÜRLER'S DISEASE AND NORMAL TISSUES

CASE*	TISSUE	TOTAL CONTENT OF STORAGE SUBSTANCE (PER CENT WET WT.)	No. of Ethanol Fraction	RATIOS: Glucuronic Acid/Glucosamine†	RATIOS: S/N‡	RATIOS: Acetyl/N	SPECIFIC ROTATION $[\alpha]_D^{25°}$ (DEGREES)
K. N.	Autopsy liver	1.4	I-A	1.50	2.00	..	+60.2§
			I-B	1.47	1.0	..	+79.6§
			I-AP-ED	1.37‖	1.00#	0.67#	....
			I-331**	1.11	1.7	0.75‡	+69.5††
	Autopsy spleen	0.11	I-S-A	1.30	1.7	..	....
			I-S-C	1.44	2	..	....
G. H.	Autopsy liver	0.4	II-D	1.35	0.66#	0.62#	+57.0§
D. C.	Biopsy liver	0.9	III-GR	1.29	1.31	0.73#	+35.8††
Normal	Autopsy liver	*Ca.* 0.01‡‡	....	..	..	..	....

* The author is grateful to Dr. C. H. Carter, Florida Farm Colony, Gainesville, Florida, for the tissue from K. N. (female, age 14 years at death); to Dr. B. H. Landing, Children's Hospital, Cincinnati, Ohio, for the tissue from G. H. (female, age 2½ months at death); and to Dr. Gilbert B. Forbes, University of Rochester School of Medicine, Rochester, New York, for the tissue from D. C. (male, age 6 years).

† Uronic acid analyzed by carbazole method (see text) and calculated as glucuronic acid. Glucosamine determined as described in the text.

‡ Nitrogen content calculated from glucosamine content.

§ Determined on aqueous solution of the polysaccharide as the free acid.

‖ Glucosamine content calculated from Dumas N determination (see Table 3).

Nitrogen by Dumas determination (see Table 3).

** Purified by Dowex-1 (formate) column chromatography, followed by ethanol precipitation of calcium salt.

†† Determined on aqueous solution of the calcium salt of the fraction.

‡‡ Obtained from sum of hexosamine contents of all fractions, using an estimated value of 35 per cent for the hexosamine content of the material. The identity of these fractions with any of those from the pathological tissues has not been established.

Identification of Constituent Sugars.—All fractions were hydrolyzed in 4 *N* HCl for 16 hours at 100°C., and the hydrolyzates were analyzed quantitatively for hexosamine by a modification[4] of the Elson-Morgan method. The liberation of hexosamine was found to be complete under these conditions of hydrolysis. In addition, qualitative information has been obtained about the kind of hexosamine present through examination of the hydrolyzates by paper chromatography, using the *n*-butanol–pyridine–water solvent (3:2:1.5) of Jeanes *et al.*,[5] by which glucosamine and galactosamine are well separated. All fractions from all cases had glucosamine as the only hexosamine present. The possible presence of other hexoses in the fractions was investigated by paper chromatography of hydrolyzates prepared in 1 *N* HCl for 3 hours at 100°. In addition, enzymatic assays were done for glucose on these hydrolyzates via the hexokinase–glucose-6-phosphate dehydrogenase–TPN system and, after addition of phosphomannose isomerase, for mannose. By these procedures, the only other sugar found in some fractions was glucose. This was shown to arise in every case from a contamination of the mucopolysaccharide fraction by glycogen, since the glucose content fell to a very low value after enzymatic degradation of 98 per cent of the residual glycogen by phosphorylase and amylo-1,6-glucosidase. Fucose was found to be absent from the mucopolysaccharide by the test of Dische and Shettles.[6]

The only constituent sugar of the mucopolysaccharide other than glucosamine was found to be uronic acid, through use of the carbazole test of Dische[7] as well as by the orcinol test of Khym and Doherty.[8] The molar ratio of apparent glucuronic acid to glucosamine was found to be from 1.3 to 1.5 with the former and

from 0.7 to 0.8 with the latter method.[9] Thus the observed ratio of apparent uronic acid content by the carbazole method to that by the orcinol method is about 2. Hoffman, Linker, and Meyer[10] have found that this ratio is 0.5 to 1 for three types of chondroitin sulfates. We have found that for heparin this ratio is about 4. The question of the type of uronic acid present was studied by subjecting the mucopolysaccharide fractions to partial hydrolysis in 1 *N* HCl at 100°. At this concentration of acid the polysaccharide is cleaved only slowly to smaller fragments. After either 7 hours or 16 hours of such hydrolysis, the only free uronic acid found on a paper chromatogram developed in the solvent of Fischer and Dorfel[11] was glucuronic acid.

The sulfate content of the mucopolysaccharide fractions was determined on 16-hour, 4 *N* HCl (100°) hydrolyzates, using a modification of the method of Dodgson and Spencer[12] for the precipitation and washing of benzidine sulfate, followed by the method of Andersen[13] for the estimation of the benzidine content of the precipitates by spectrophotometric observation at 249 mμ. All fractions were found to be sulfated. After purification the fractions showed ratios of sulfur to nitrogen of either 0.66, 1.0, 1.33, 1.67, or 2.0.

The possible presence of acetyl groups was investigated by *p*-toluene sulfonic acid hydrolysis of the polysaccharide, followed by distillation of acetic acid under reduced pressure according to the method of Elek and Harte.[14] Several samples of the polysaccharide were also analyzed for acetyl groups by Dr. Adalbert Elek. A uniform finding was the presence of a considerable acetyl content in all samples analyzed, the molar ratio of acetyl to nitrogen being 0.67. A study of the yield of acetic acid as a function of time of reflux with toluene sulfonic acid showed that the analysis was performed under conditions of maximum acetyl yield.

Structural Features.—The question of the nature of the linkage of the sulfate to the polysaccharide chain was studied by measuring the sulfate released on mild acid hydrolysis of a fraction with a sulfur-to-nitrogen ratio of 1.0. Approximately one-third of the total sulfate was hydrolyzed in 30 minutes at 100° in 0.04 *N* HCl. After 60 minutes, from 43 to 57 per cent (in duplicate experiments) of the total sulfur had been released as inorganic sulfate, and similar values were obtained after 1 hour and 3 hours of additional hydrolysis. These data suggest that a part of the sulfur may be present in the intact polysaccharide in a sulfamic-type linkage to the nitrogen of some of the glucosamine residues. Jorpes *et al.*[15] have shown that this bond occurs in heparin. In the case of the mucopolysaccharide fractions from Hürler's syndrome, the colorimetric amino-N test of Frame *et al.*[16] was applied to the intact polysaccharide (fraction I-AP-ED, Table 1) and to samples hydrolyzed in 0.04 *N* HCl as discussed above. It was found that the intact polysaccharide gave some color in this test but that the apparent amount of amino-N rose to about three times this value after 15 minutes of hydrolysis and remained unchanged after 2 hours of hydrolysis. It was impossible to calculate the percentage of the total nitrogen set free as amino-N, because of a marked hyperchromic effect of the structure of the polysaccharide on the color yield of the amino groups of the glucosamine residues present in the chain. The fact that there appears to be a concomitant appearance of free amino groups under mildly acidic hydrolysis conditions when inorganic sulfate is also set free supports the suggestion that in the Hürler disease storage substance some of the glucosamine residues have acetylated amino

groups (see above), while others have their amino groups bound in amidosulfonic acid linkage.

Early in the work it was observed that the mucopolysaccharide fractions were slowly dialyzable through Visking sausage casing and were more rapidly electrodialyzable. Brief electrodialysis was occasionally used to prepare the materials in the form of their free acids for analytical study. The relative ease of dialysis suggested that the average size of the molecules is rather small—and, accordingly, aqueous solutions of several fractions which had been extensively dialyzed and, in some cases, electrodialyzed were subjected to physical study in the Model E Spinco analytical centrifuge. A synthetic boundary cell was used, and sedimentation constants and diffusion constants were calculated from the same pictures. In addition, diffusion constants were calculated from runs made at low speed, after it became apparent that the material under study had a very high diffusion constant and a very low sedimentation constant. Partial specific volumes were determined pycnometrically and in a density-gradient tube. The results of these physical studies are shown in Table 2. The molecular weights calculated from

TABLE 2

PHYSICAL PROPERTIES OF THE STORAGE SUBSTANCE FROM LIVER

FRACTION*	ELECTROPHORETIC MOBILITY AT $\Gamma/2 = 0.1$ ($\times 10^{-5}$ CM^2/VOLT/SEC) Acetate (pH 5.0)	Veronal (pH 8.6)	SEDIMENTATION CONSTANT, $S_{20,w}$ (SVEDBERG UNITS)	DIFFUSION CONSTANT, $D_{20,w}$ ($\times 10^{-7}$) CM^2/SEC	PARTIAL SPECIFIC VOLUME	CALCULATED MOLECULAR WEIGHT
I-B	−15.8	−15.6	..	..	..	..
I-A	....	....	0.92	20	0.46	2075
I-AP-ED	....	....	0.76	24.5	0.44	1350
II-D	....	....	0.66	28.3	0.53	1240

* See Table 1.

sedimentation-diffusion data are probably accurate within 20 per cent. These values, as well as the observations on dialyzability mentioned above, suggest that the storage substance might more properly be said to belong to the class of oligosaccharides than to that of the polysaccharides. Each of the fractions examined in the ultracentrifuge sedimented as a single peak. Further, a plot of the apparent diffusion constant versus time was linear and had only a small slope. These criteria speak for the relative homogeneity of the fractions. On the assumptions that each substance is pure, that the materials have generally similar structure, but that they differ from each other chiefly in their degree of sulfation (see Table 1), the storage oligosaccharides appear to consist of seven monosaccharide units. Further discussion of the detailed structure of the substances is given below.

The oligosaccharide storage substances from the livers of the three cases of Hürler's disease resemble heparin in many of their structural features: (1) in being composed exclusively of glucosamine and glucuronic acid units; (2) in being sulfated and apparently having some of the sulfate groups present in amidosulfonic acid linkage; (3) in having a considerable positive optical rotation. The materials differ from heparin in having acetyl groups present, presumably as N-acetyl, and in being of low molecular weight. Assays for anticoagulant activity were made on fractions I-A and I-B by the method of Blomback *et al.*[17] It was found that these substances had, if any, less than 2 per cent of the activity of heparin[18] on a weight basis.

Partial acid hydrolyzates of fraction I-AP-ED and of heparin were prepared in 1 *N* HCl at 100° for 7 hours and for 16 hours. After removal of chloride ion with Ag_2CO_3, the hydrolyzates were examined by paper chromatography, using the pyridine–ethyl acetate–acetic acid–water (5:5:1:3) solvent system of Fischer and Dorfel.[11] Figure 1 shows such a chromatogram. The storage oligosaccharide is

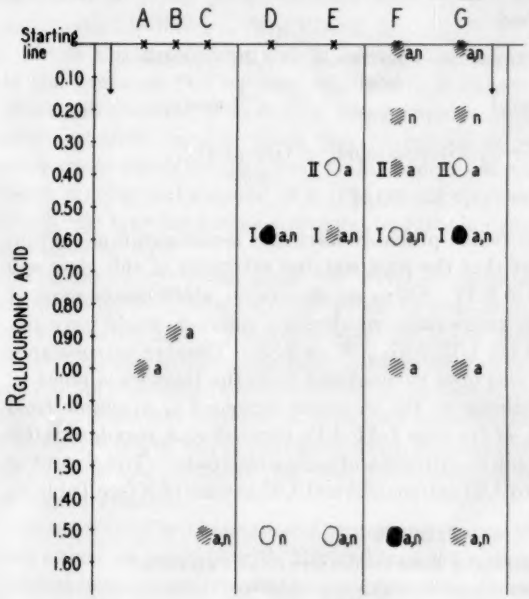
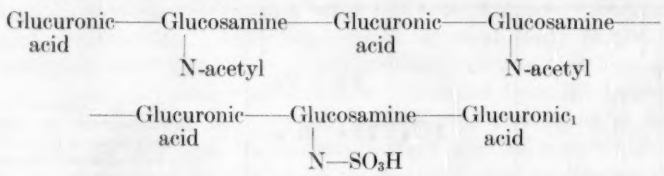


FIG. 1.—Descending paper chromatogram of partial acid hydrolyzates of heparin and of a Hürler's disease oligosaccharide fraction. Strip *A*, glucuronic acid; *B*, galacturonic acid; *C*, glucosamine; *D*, heparin, 7 hours, 1 *N* HCl; *E*, heparin, 16 hours, 1 *N* HCl; *F*, fraction I-AP-ED, 16 hours, 1 *N* HCl; *G*, fraction I-AP-ED, 7 hours, 1 *N* HCl. The relative intensity of the sprayed spots is indicated by the degree of shading. Reaction with an aniline phthalate spray is indicated by *a;* reaction with a ninhydrin spray is indicated by *n*.

similar to heparin in yielding a large amount of the same substance, I, at 7 hours. As shown, the amount of this material was markedly reduced in both cases by further hydrolysis. It is interesting that this fragment appears to terminate on its reducing end with a glucosamine unit, since it reacts with a ninhydrin spray. Such a product would not be expected as a principal component of a partial acid hydrolyzate of heparin in the light of the present ideas about the structure of this polysaccharide. On the other hand, the type of structure postulated below for the storage oligosaccharide would be expected eventually to yield a considerable amount of a disaccharide terminating in glucosamine during acid hydrolysis. Figure 1 also shows that heparin and the storage oligosaccharide are alike in yielding a substance, II, whose amount increases with increasing time of hydrolysis. This fragment, more abundant from the storage oligosaccharide than from heparin, appears to terminate in a glucuronic acid unit at its reducing end. Other substances having lower mobility on paper, and apparently terminating in glucosamine units, were detected in the hydrolyzate of the storage oligosaccharide. Although free glucosamine was present in all hydrolyzates, its amount was considerably greater in those of the storage oligosaccharide than in those of heparin. This is probably due partly to the lower molecular weight of the former substance but chiefly to the presence of N-acetylated glucosamine residues whose hexosaminidic

bonds are cleaved first by acid hydrolysis, followed by liberation of free glucosamine. A distinct feature of the chromatograms from the oligosaccharide was always seen to be the presence of glucuronic acid at all intermediate stages of hydrolysis. No glucuronic acid could be detected from heparin.

It is believed that the observations made can be unified somewhat by postulating that the storage oligosaccharides often are heptasaccharides to which the following partial structure may be assigned:



Most or all of the glycosidic bonds probably have the α-configuration. From this structure one would predict that the least sulfated substance of this class will have a sulfur-to-nitrogen ratio of 0.33. Other substances, in which one or more of the free hydroxyl groups of the glucosamine residues are sulfated, would have sulfur-to-nitrogen ratios of 0.66, 1.00, 1.33, 1.67, 2.00, or 2.33. Thus far, no substance with a ratio of 0.33 or of 2.33 has been encountered from the tissues examined.

Additional information pertaining to the structure proposed is available from the titration curve of 1.92 mg. of fraction I-AP-ED, titrated with standard 0.008 *N* NaOH (CO_2-free) at 27°, using N_2 stirring and a glass electrode. This weight of electrodialyzed sample contained 3.63 μatoms of S and 3.67 μatoms of N (see Table 3).

TABLE 3

ELEMENTARY COMPOSITION OF SOME OLIGOSACCHARIDE FRACTIONS*

ELEMENT OR GROUP	FRACTION II-D (Mol. Wt. 1450) (Per Cent) Calc.†	II-D Found	I-AP-ED (Mol. Wt. 1530) (Per Cent) Calc.‡	I-AP-ED Found	I-331 (Mol. Wt. 1766) (Per Cent) Calc.§	I-331 Found
C	..	..	35.79	35.62	..	..
H	..	..	4.61	5.27	..	..
N	2.90	3.01	2.72	2.68	2.38	2.28‖
S	4.42	4.54	6.23	6.07	9.07	9.16
Acetyl	5.94	5.74	5.58	5.47	4.87	5.25

* Microanalyses by Dr. Adalbert Elek, Los Angeles, California. All analyses on samples dried to constant weight at 78° *in vacuo* over P_2O_5. The analyses found have been corrected for a small contamination by glycogen determined microenzymatically as glucose (see text).

† Calculated for the free acid form of a heptasaccharide with S:N = 0.66 (see text and Table 1).

‡ Calculated for the free acid form of a heptasaccharide with S:N = 1.00 (see text), correcting for the presence of 1.6 per cent ash as K. Sample was electrodialyzed before analysis.

§ Calculated for the calcium salt (2 Ca) of a heptasaccharide with S:N = 1.67 (see text and Table 1).

‖ Calculated from glucosamine analysis.

From its ash content (1.6 per cent), it can be calculated that it contained 0.45 μatom of cation (calculated as K). The titration curve showed the consumption of 4.90 μmoles of NaOH from a small rise at pH 2.97 (corrected to zero volume change) to the major rise at pH 6.50. This quantity of NaOH may be taken to measure the glucuronic acid carboxyl groups. The midpoint of their titration (corrected to zero volume change) was at pH 3.45 (pK′). The consumption of NaOH for their titration indicates that the number of carboxyl groups is 1.33 times as great as the number of glucosamine residues. The heptasaccharide structure assigned has such a ratio.

Table 3 gives analytical data on the elementary composition of some of the oligosaccharide fractions from two of the cases of Hürler's disease which have been studied. The composition of each has been calculated with reference to a heptasaccharide having the structure given above and with a varying number of sulfate groups as indicated. The molecular weight of the proposed heptasaccharide structure is shown for comparison with the molecular weights calculated from sedimentation-diffusion data (Table 2).

While this manuscript was in preparation, a report appeared[19] on the finding in the urines of two patients with Hürler's disease of a considerable amount of chondroitin sulfate B and of a small amount of a poorly characterized mucopolysaccharide fraction which may be related in structure to the storage oligosaccharides which are described in this paper. Meyer has reported[20] on the occurrence in aorta and amyloid of a type of mucopolysaccharide ("heparitin sulfate") which also may have some structural features in common with the oligosaccharides described herein.

The structure of the family of sulfated oligosaccharides accumulated in the tissues of patients with Hürler-Pfaundler's disease is only partially clarified by the work reported here. Further studies are under way to extend this information. It is impossible to state at present whether these substances of low molecular weight are normal constituents of human tissues, present in very small amounts, or whether they are abnormal products of synthetic mechanisms peculiar to Hürler's disease. The three cases of this disease from which tissues have been available were found to be qualitatively similar in that they all possessed a large amount of this new class of oligosaccharides.

Summary.—The characteristic storage substance which accumulates in the liver and spleen of patients with Hürler-Pfaundler's disease ("gargoylism") has been isolated from tissues removed at autopsy and by biopsy. By suitable fractionation procedures, this water-soluble material has been shown to consist of a family of oligosaccharides each of which is composed exclusively of D-glucosamine and D-glucuronic acid units united in glycosidic linkage. Two-thirds of the glucosamine residues of the molecule are acetylated, and the remaining one-third most probably is united with sulfate in an amidosulfonic acid linkage. The oligosaccharides are all further sulfated and differ from one another in the degree of this sulfation. The substances are of low molecular weight, as shown by their slow dialyzability and by calculation from determinations of sedimentation and diffusion constants. Elementary analyses support the assignment of a heptasaccharide structure to many of the fractions studied. In such a structure the ratio of glucuronic acid to glucosamine residues is 1.33. The substances all have a considerable positive optical rotation. One possible detailed structure of the molecule is discussed.

* A preliminary report of these findings was made at the Conference on the Metabolism of Mucopolysaccharides held at the Retina Foundation, Boston, Massachusetts, June 1 and 2, 1956. This work has been supported in part by a grant (RG 4761) from the National Institutes of Health, United States Public Health Service.

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[9] In addition to the fractions shown in Table 1, a substance having an apparent glucuronic acid-hexosamine ratio of 0.62 by the carbazole method was isolated in the amount of 0.15 per cent of the wet weight from the spleen of case K. N. The nature of the hexosamine in this fraction was not investigated. Presumably, this polysaccharide belongs to a different series of compounds; it may be structurally related to chondoitin sulfate B.

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*VACCINE FOR THE PREVENTION IN HUMANS OF COLDLIKE SYMPTOMS ASSOCIATED WITH THE JH VIRUS**

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Communicated by K. F. Maxcy, March 11, 1957

The discovery of the adenoviruses[1, 2] constituted a major advance in leading to the identification of agents responsible for common respiratory disease of presumed viral etiology in the human population. These agents have been shown to be of particular importance in military populations.[3–4] However, studies from this laboratory[5] as well as from others[7, 8] have indicated that these agents produce little clinical disease in the civilian populations studied, although the significance of the adenoviruses in causing disease in infants and children has not yet been determined.

In agreement with the findings of Dingle and co-workers,[9] it has been our experience that respiratory illness makes up about 70 per cent of all illnesses seen in families and various groups we have studied.[6] No etiologic agent has as yet been isolated which accounts for the major share of such illnesses, which clinically are of the common-cold variety.

In a recent paper[10] from this laboratory, evidence was presented for the isolation of a new virus (JH) which was associated with mild upper-respiratory illness in humans. In this paper we wish to report the development of a vaccine which protects individuals against the coldlike symptoms associated with the JH virus.

MATERIALS AND METHODS

Virus Isolations.—The JH virus was isolated in rhesus monkey kidney epithelial tissue cultures as described previously.[10] The cultures were inoculated when 5 days old and were maintained in 199 medium (Difco) at 36.5° C., also as described earlier.[10]

Neutralization tests with the JH virus were carried out, using approximately 500 TCD_{50} of the virus. A fourfold rise was considered significant. Control uninfected cultures were included in all tests. Neutralization tests were used because no reliable complement-fixation test has yet been worked out for this virus.

Examination of Children.—All children were examined daily, beginning on the third day of the outbreak, and temperatures were taken in the morning and in the evening. All children participated in this work with the written consent of their parents.

Vaccine Preparation.—The JH isolate used to prepare the vaccine was originally isolated from a child with coryza, mild sore throat, and a fever of 99.9° F. It was isolated from nasal washings inoculated into monkey kidney epithelial tissue cultures as described previously.[10] The isolate had been passed ten times in monkey kidney and had a titer of $10^{7.1}$ TCD_{50}/0.1 ml. at its tenth passage.

The virus suspension used to prepare the vaccine was tested by the methods described in the previous paper,[10] to rule out the possibility of other cytopathogenic agents being present in the JH virus suspension. The cytopathogenic effects caused by the JH virus suspension were completely inhibited if this suspension was mixed with antisera prepared against the JH virus, using other isolates of JH virus to prepare the antisera.

The infected monkey kidney epithelial cultures were harvested 8 days after the addition of the JH virus. At the same time that infected cultures were prepared, one-half of the culture tubes were left uninoculated to serve as controls and for the preparation of a placebo. Most of these tubes were harvested at the same time as the infected tubes and treated in the same manner. Ten of the control tubes were observed for 14 days. At this time no cytopathogenic effects were detectable. Two blind passages of the control suspensions were made into monkey kidney cultures, and no cytopathogenic effects were observed when these cultures were grown in 199 medium or in a medium containing 2 per cent calf serum and hydrolyzed lactoalbumin or in the serum medium described previously.[10] Ten tubes in each medium were observed for 14 days. These results indicate that no detectable simian viruses were present in the monkey kidney cultures used to prepare the vaccine.

After harvest of the infected cultures, the contents of the tubes were pooled and homogenized in a Waring Blendor. The suspension was centrifuged at 2,000 rpm in a small desk International centrifuge, and the supernatant fluid was then filtered through a sintered glass filter of medium porosity. The addition of antisera to the JH virus, prepared with other JH isolates, completely inhibited the vaccine preparation from causing detectable cytopathogenic effects when inoculated into monkey kidney tissue culture and observed for 10 days. Titrations showed that the virus preparation used for these neutralization tests contained approximately $10^{3.5}$ TCD_{50}.

Just before inactivation, the filtered JH virus preparation contained $10^{6.9}$ TCD_{50}.

In order to inactivate the material, a final concentration of 1:2,000 formalin was added to the solution. This preparation was held at 35° C. for 7 days, occasionally being shaken by hand. After this time the formalin-treated virus solution was dialyzed (with constant stirring) against 199 solution at 3.5° C. to get rid of free formaldehyde. After the dialysis procedure, which by itself did not significantly lower the infectivity of live JH virus, fifteen monkey kidney culture tubes containing 0.9 ml. of 199 medium were inoculated with 0.2 ml. of the dialyzed virus preparation. No detectable cytopathogenic effects were observed in 18 days, and two blind passages also failed to show evidence of cytopathogenic effects. The vaccine was stored in sterile bottles at 3.5° C.

Safety Tests.—Many safety tests were carried out. Just before the JH virus was treated with formalin, the following media were tested with aliquots of the preparation: trypticase soy agar, blood agar, tryptose agar, serum ascites agar, Lowenstein's medium, Brewer's trioglycollate broth, and Sabouraud's agar. None of the cultures showed growth of any kind when they were incubated under anaerobic and aerobic conditions at 36.5° C. or room temperature for 3 weeks. The following animals were inoculated with the virus preparation just before it was inactivated: rhesus monkeys intracerebrally and intramuscularly; rabbits intracerebrally, intraperitoneally, and intracutaneously; adult mice intracerebrally; suckling mice intracerebrally and intraperitoneally; hamsters intraperitoneally and intranasally; and guinea pigs intraperitoneally. The animals were observed for 35 days. During this time there was no fever or other sign of clinical illness. Histopathological examination of the brain and cord of the inoculated monkeys showed no sign of infection at the end of the observation period. The same safety tests were repeated on the inactivated JH virus preparation after it had been stored in the bottles for 2 days as described above. Again, all results were negative. The uninfected monkey kidney cultures were treated in the same manner as described for the infected preparation. In the safety tests at least five animals of each species were used for each vaccine or control preparation in the case of the large animals, and at least fifteen animals in the case of the small rodents.

RESULTS

Design of Vaccine Test.—Our previous experience[10] with the JH virus had shown that it can be associated with mild upper-respiratory outbreaks in the human population. Therefore, fairly large groups of individuals were given either placebo or vaccine injections. In picking the individuals who received the various injections, the following procedure was followed. The names of the individuals to be inoculated were written on small slips of paper and dropped into a hat. The slips were then picked out of the hat. Every other name selected from the hat was given vaccine. The groups that were selected to be vaccinated were picked on the basis that the individuals making up these groups lived in the same building, had intimate contact with each other, and were of the same age, sex, and race. Thus, if a respiratory outbreak occurred in the group, such variables as exposure to the agent, age, sex, race, and environment could be controlled to some extent.

The physicians who examined the groups did not know which individuals in the groups received vaccine and which received placebos. The workers doing the antibody determinations against the JH virus had no idea as to the source of their

serum samples, since at that time serum samples from infections due to adenoviruses and influenza were also being tested against the JH virus as part of the general respiratory study being carried out in the laboratory.

Effect of JH Vaccine on Respiratory Outbreak.—Although a number of groups were vaccinated and given placebos, only the results of one group will be discussed in this paper, because it was the only group which experienced a respiratory outbreak associated with the JH virus.

The individuals in the group consisted of 114 children between the ages of ten and fourteen. They lived and slept in one large dormatory. This group had been broken up into three parts. All individuals in each of the groups were distributed randomly in the cottage. The 50 children making up the first group were given 1.0 ml. of vaccine intramuscularly and were given another injection 1 month later. Twenty-five children were given two injections of the control uninfected preparation, and 25 were given two injections of saline. These injections were given in the same manner as the vaccine. All the children were bled before being given their first injection. Unfortunately, because of a mixup in the bleeding schedule, the children were not bled again until 2 weeks after their second injection. They were bled again 2 weeks later. Table 1 shows that children receiving the vaccine

TABLE 1

NEUTRALIZING ANTIBODY RESPONSE TO THE JH VACCINE

Serum Specimen	Prevaccination Titer	Postvaccination Titer*
1-R	0†	1:8
3-R	1:4	1:64
5-R	0	1:16
7-R	1:8	1:128
9-R	0	1:16
11-R	1:4	1:64
13-R	0	0
15-R	0	1:64

* Sera taken 2 weeks after second injection.
† 0 means less than 1:4.

responded with neutralizing antibodies to the JH virus. Ten children given the uninfected preparation and 10 children given saline showed no increases in their neutralizing antibody titer to the JH virus. All these determinations were carried out on the sera collected 2 weeks after their second injection.

The respiratory outbreak occurred $5^1/_2$ weeks after the second injection. It was characterized by coryza, mild sore throat, and fever not over 100° F., and in some cases a cough. It lasted about 2 weeks, with the great majority of the cases occurring at the beginning of the second week. The duration of illness was 4 days. The routine clinical blood examination was normal, as was a throat culture taken on any individual showing overt illness. Table 2 shows the relationship of the various clinical symptoms in the total number of overt cases that occurred during the outbreak. Three of the 26 children showing overt illness had a fever between 102 and 103 degrees for about 2 days. The other sick children had fevers of 100 degrees or less.

Tests to show the etiology of this outbreak were negative for streptococcus, adenoviruses, Coxsackie viruses, and influenza, using the methods described previously.[10] However, two of eleven of the individuals showing overt disease yielded JH virus in their nasal washings. Because of the difficulty in isolating the

TABLE 2

RELATIONSHIP OF VARIOUS SYMPTOMS ASSOCIATED WITH THE RESPIRATORY OUTBREAK

Symptoms	No. of Patients Showing Various Clinical Combinations*	No. of Patients Showing Antibody Rise to JH Virus
Coryza, mild sore throat, slight fever, cough	$^{7}/_{26}$**	$^{7}/_{7}$†
Coryza, mild sore throat, slight fever, no cough	$^{13}/_{26}$	$^{9}/_{13}$
Coryza and slight fever, no cough or sore throat	$^{3}/_{26}$	$^{2}/_{3}$
Coryza and slight sore throat, no fever or cough	$^{3}/_{26}$‡	$^{2}/_{3}$

* Denominator is total number of patients showing overt disease, and numerator is number of patients showing indicated clinical symptoms.

† Denominator is total number of patients showing various clinical relationship, and numerator is number of these showing significant neutralizing antibody rises to JH virus. Convalescent sera collected 4 weeks after illness.

‡ One of these patients received the vaccine.

** Three of these children showed fevers between 102 to 103 degrees.

JH virus with the techniques available at the present time, it is hard to assess the role of this agent in causing disease by this method. However, the serologic response to the JH virus reported in the previous paper,[10] and the great specificity of this response,[10] makes such a test more feasible in trying to determine the importance of this agent in causing human illness. From Table 3 it can be observed

TABLE 3

OVERT RESPIRATORY ILLNESS IN INDIVIDUALS RECEIVING INACTIVATED JH VACCINE AND PLACEBO

Injection	No. Showing Overt Respiratory Illness	No. of Patients Showing Significant Antibody Rise to JH Virus*
Saline	$^{11}/_{25}$†	$^{9}/_{11}$
Control uninfected preparation	$^{12}/_{25}$	$^{9}/_{12}$
Vaccine	$^{3}/_{50}$	$^{2}/_{3}$

* Fourfold increase in neutralizing antibody was considered significant. Convalescent sera collected 4 weeks after illness.

† Denominator is total number of people comprising each group, and numerator is number of people developing overt respiratory disease during outbreak.

that, of 26 individuals showing overt disease, 20 showed antibody rises to the JH virus. It is felt, therefore, that the JH virus may have been responsible for this outbreak. This view is greatly strengthened by the results which showed that, of the 50 children receiving the vaccine, only 3 developed respiratory illness during this outbreak, whereas 23 children out of 50 who did not receive the vaccine developed overt disease. The sera of 3 of 15 children tested who received no vaccine and showed no overt illness during this outbreak showed significant increases in neturalizing antibody to the JH virus. Thus, on the basis of this very small sample, 20 per cent of the children experienced subclinical infection with the JH virus in this outbreak. It is realized that this number is far too small to permit any significant conclusions about the subclinical attack rate of the JH virus.

DISCUSSION

The data presented in the previous paper[10] and the observation in this paper that an inactivated vaccine prepared from the JH virus will reduce the incidence of mild upper-respiratory illness about eightfold, in a respiratory outbreak with which the JH virus was found to be associated, indicate that the JH virus is capable of causing a coldlike disease in the human population.

The data indicate that an effective and safe vaccine can be prepared from inactivated JH virus against an infection which clinically is very similar to the common cold. Two major questions that remain to be answered are the length of time the protective antibodies produced by the killed JH virus vaccine will last and the optimal spacing of the vaccine injections in order to give the greatest and longest-lasting protection.

The most direct way in which the JH virus could be shown to be responsible for the coldlike symptoms with which it is associated would of course be to inoculate human volunteers and show that, under controlled conditions, the JH virus produces the clinical respiratory illness. Unfortunately, we were not able to carry out such an experiment. However, it should be pointed out in this connection that the failure of the JH virus to produce the overt disease in volunteers does not necessarily mean that it is not the etiologic agent. In view of the great difficulty in isolating this agent, it is quite possible that only a few virus particles present in the total JH virus population are capable of growing in monkey kidney epithelial tissue cultures. The viruses that reproduce might be avirulent for humans. It is therefore felt that, while the human-volunteer experiment should be done, the vaccine experiments reported in this paper are direct proof that the JH virus is capable of causing coldlike symptoms in humans.

SUMMARY

1. The JH virus was found in association with an upper-respiratory outbreak in children.

2. Children given a vaccine prepared from inactivated JH virus showed an attack rate about eight times lower than that of the children receiving the placebo injections.

3. No untoward reactions were observed in 401 individuals receiving the vaccine, which was prepared from inactivated JH virus grown in monkey kidney epithelial tissue.

4. It is concluded that the JH virus is the cause of the coldlike illness in humans and that an inactivated vaccine can be prepared from this virus which protects against the overt illness due to the JH virus.

* This work was supported by a grant from the Rockefeller Foundation. The JH virus is not related to the croup virus, to the two chimpanzee rhinitis viruses, or to Sendai virus, on the basis of cultural, growth, and serological properties, or to any of the other viruses mentioned in in reference 10 below.

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*OCCURRENCE OF ORNITHINE δ-TRANSAMINASE: A DICHOTOMY**

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Against the background of the biochemical unity (cf. Kluyver and van Niel[1]) of all forms of life, instances of "biochemical diversity" have held a persistent attraction for students of biochemistry and physiology and of taxonomy and evolution. One striking example of such diversity can be seen in the dissimilar modes of ornithine synthesis in *Neurospora crassa* and *Escherichia coli*:[2] in *N. crassa* ornithine arises by transamination of glutamic γ-semialdehyde,[3] whereas in *E. coli* ornithine synthesis proceeds via several acetylated intermediates.[4]

The differences between the two ornithine pathways are reflected in the results of enzymatic studies: an enzyme that mediates the reversible transamination between glutamate and glutamic γ-semialdehyde could be demonstrated in extracts of *N. crassa*[5, 6] (and of some other micro-organisms[6]) but not in those of *E. coli*.[6] The enzyme from *N. crassa* has been named "ornithine δ-transaminase," after the reverse of the ornithine-yielding biosynthetic reaction (see Fig. 1).

The differences in the mode of ornithine synthesis and in ornithine δ-transaminase activity between *N. crassa* and *E. coli* encouraged the present, more systematic, study of the distribution of this enzymatic activity. Attention was initially focused on various fungi and eubacteria; subsequently some other organisms of interest were included in the survey.

The organisms were selected partly on the basis of their taxonomic position and partly on the basis of their ability to grow on simple, defined media (free of ornithine or glutamic γ-semialdehyde). Accordingly, to the extent that such media were used, this study is concerned with constitutive, rather than adaptive, ornithine δ-transaminase activity.

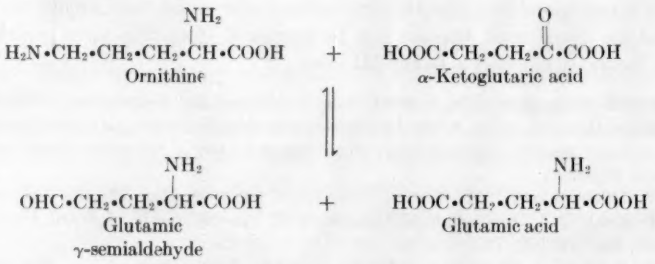

FIG. 1. Reaction catalyzed by ornithine δ-transaminase

For the survey of this activity, the organisms employed were appropriately disrupted, and the clarified cell-free extracts obtained were subjected to a diagnostic color reaction and bioassay.

MATERIALS AND METHODS

Organisms.—The strains identified by ATCC numbers were obtained from the American Type Culture Collection (Washington, D.C.). *Saccharomyces cerevisiae*

(Fleischmann's bakers' yeast, Standard Brands, Inc.), spinach leaf, and calf liver came from commercial sources. The remaining organisms were kindly provided, as indicated below, by various individuals, to whom the authors are very greatly indebted. *Micrococcus lysodeikticus* was received from Dr. L. Gorini, *Bacillus subtilis* from Mrs. M. Bonner, *B. megaterium* from Dr. P. B. Cowles, *Mycobacterium ranae* from Dr. M. I. Bunting, *N. crassa* from Dr. P. St. Lawrence, *N. sitophila* (thiamine-requiring mutant[7]) from Mrs. M. Bonner, *Coprinus lagopus* from Dr. H. Papazian (who isolated this organism), *Chlamydomonas reinhardi* (isolated by Dr. G. M. Smith) from Dr. W. T. Ebersold, *Chlamydomonas moewusii* (isolated by Dr. L. Provasoli) from Dr. R. R. L. Guillard, *Tetrahymena pyriformis* from Dr. G. W. Kidder, *Hydrogenomonas ruhlandii*[8] from Dr. L. Packer, *Pseudomonas fluorescens*[9] from Dr. W. Vishniac, *E. coli* strain B and *Aerobacter aerogenes* from Mrs. M. Bonner, *Serratia marcescens*[10] from Dr. M. I. Bunting, *Proteus vulgaris* from Mrs. M. Bonner, *Salmonella enteritidis* from Dr. A. D. Mandel, and *Anacystis nidulans*[11] from Dr. J. Myers.

Media.—With the exception of *Saccharomyces cerevisiae* and of *T. pyriformis* (see below), the organisms used were grown on the following chemically defined media: (*a*) Gorini and Lord's medium[12] containing glucose, acetate, glutamate, biotin, and salts; (*b*) Medium E[13] supplemented with glucose (0.2 per cent) and biotin (2.5 μg/liter);[14] (*c*) Medium E[13] with glucose (0.5 per cent); (*d*) Medium E[13] with glycerol (2.0 per cent); (*e*) Medium E[13] with sucrose (2.0 per cent); (*f*) Medium N with sucrose (2.0 per cent);[15] (*g*) Fries medium[16, 17] supplemented with sucrose (2.0 per cent) and thiamine (3.0 μg/ml); (*h*) glucose-glutamate-thiamine-salt medium, kindly communicated by Dr. H. Papazian; (*i*) Sager and Granick's medium;[18] (*j*) Guillard's medium;[19] (*k*) proteose-peptone (3.0 per cent) and glucose (0.5 per cent), as recommended by Dr. G. W. Kidder, whose helpfulness and advice in this connection are gratefully acknowledged; (*l*) Medium E[13] with sodium lactate (0.5 per cent); (*m*) Medium Z with sucrose (1.5 per cent);[20] (*n*) Medium E[13] with sodium succinate (0.5 per cent); (*o*) Labrum and Bunting's synthetic medium;[10] (*p*) Medium E[13] supplemented with glucose (0.25 per cent) and nicotinic acid (0.25 μg/ml); (*q*) Medium C of Kratz and Myers[11] with sodium succinate (0.5 per cent) and carbon dioxide (0.5 per cent in air, volume by volume).

Cultivation of Organisms and Preparation of Extracts.—The conditions used for the cultivation of the organisms are indicated in Table 1.—After cultivation, the organisms were harvested by centrifugation, except *T. pyriformis*, which was allowed to sediment by gravity. The organisms thus collected were washed in chilled 0.1 *M* phosphate buffer (pH 7.0) and resuspended and disrupted in the same buffer at 0°–5° C.

All the bacteria used, except *M. lysodeikticus* and *Arthrobacter histidinolovorans*, were disrupted by sonic vibration (applied for 30 minutes) in a 9-kc. Raytheon oscillator. These two bacteria and all the remaining organisms (except *T. pyriformis*, which was ground in the frozen state without abrasive) were disrupted by grinding with alumina (A-301, Alcoa); spinach leaf and calf liver were ground with sand. The resulting extracts were clarified by cold centrifugation, dialyzed against cold 0.1 *M* phosphate (pH 7.0) when necessary, and promptly tested as sources of enzyme activity. In general, the extracts obtained could be stored at −15° C. for several weeks without detectable serious deterioration. The protein content of

the extracts was of the order of 10 mg/ml. Protein was determined by the method of Lowry *et al.*[21]

Test for Ornithine δ-Transaminase Activity.—For preliminary screening, a color test was employed. This test, which depends on the formation of the yellow color resulting from the reaction of Δ^1-pyrroline-5-carboxylate with *o*-aminobenzaldehyde,[22] was carried out in the general manner described by Fincham.[5] Δ^1-Pyrroline-5-carboxylate is the spontaneously cyclized form of glutamic γ-semialdehyde,

TABLE 1

OCCURRENCE OF ORNITHINE δ-TRANSAMINASE ACTIVITY

ORGANISM	CULTIVATION CONDITIONS: Medium*	Temperature (° C.)	Agitation	Duration (Days)	Gram Character
ACTIVITY FOUND					
Micrococcus lysodeikticus, S	*a*	25	+	3	Positive
Arthrobacter histidinolovorans, ATCC 11442†	*b*	25	+	2	Variable
Bacillus subtilis, Marburg‡	*c*	37	+	2	Positive
Bacillus licheniformis, ATCC 11560	*c*	30	+	2	Positive
Bacillus pumilus, ATCC 7061	*b*	30	+	2	Positive
Bacillus megaterium, C	*c*	30	+	2	Positive
Mycobacterium ranae, 10	*d*	37	+	2	Positive
Saccharomyces cerevisiae, Gebrüder Mayer	..	..	..	..	Positive
Torulopsis utilis, ATCC 9950	*e*	30	+	1	Positive
Neurospora crassa, 74A	*f*	30	+	2	....
Neurospora silophila, S-1090a	*g*	30	−	3	....
Coprinus lagopus	*h*	25	−	7	....
Chlamydomonas reinhardi, 137C§	*i*	25	−	6	....
Chlamydomonas moewusii§	*j*	25	−	6	....
Tetrahymena pyriformis, W†	*k*	25	+	3	....
Spinach leaf	..	..	..	..	....
Calf liver	..	..	..	..	....
ACTIVITY NOT DETECTED					
Hydrogenomonas ruhlandii‖	*l*	30	+	1	Negative
Pseudomonas fluorescens, Tr-23	*c*	30	+	2	Negative
Azotobacter agile, ATCC 9104	*m*	30	+	2	Negative
Agrobacterium radiobacter, ATCC 6466	*c*	30	+	2	Negative
Alcaligenes faecalis, ATCC 8750	*n*	37	−	2	Negative
Escherichia coli, ATCC 9637‡	*c*	37	+	1	Negative
Escherichia coli, B‡	*c*	37	+	1	Negative
Aerobacter aerogenes, Yale	*c*	37	+	1	Negative
Erwinia carotovora, ATCC 8061	*c*	30	+	2	Negative
Serratia marcescens, HY	*o*	37	+	2	Negative
Proteus vulgaris, Yale	*p*	37	+	1	Negative
Salmonella enteritidis, ETS 64	*c*	37	+	2	Negative
Anacystis nidulans§	*q*	39	+	5	Negative

* The letters in this column refer to the subsection "Media" under "Materials and Methods."

† This organism was classified as exhibiting the transaminase activity on the basis of the color reaction only, since the bioassay was obscured by the relatively large amounts of proline present in the enzyme preparations derived from this organism; the proline (found even in dialyzed preparations) appears to arise from protein breakdown.

‡ This result was unchanged when the medium used for the cultivation of this organism was supplemented with 0.2 per cent N-Z-Case (Sheffield) and 0.2 per cent yeast extract (Difco).

§ This alga was cultivated with illumination.

‖ The same result was obtained when this organism was grown autotrophically (rather than heterotrophically).

which in turn is produced through the δ-transamination or ornithine. The yellow color is due to a dihydroquinazolinium compound, which is dissociable into its original components.[22] Since the reaction with *o*-aminobenzaldehyde is not uniquely specific for Δ^1-pyrroline-5-carboxylate,[23] enzymatic reaction mixtures that showed a yellow color were subjected to bioassay with *E. coli* mutant strain 55-25. This strain gives a growth response either to Δ^1-pyrroline-5-carboxylate[22, 24] or to proline,[22] or, presumably, to proline peptides.[25] As far as is known, L-Δ^1-

pyrroline-5-carboxylate is the only compound that gives both a yellow color with *o*-aminobenzaldehyde and a growth response with strain 55-25.

The enzyme tests were carried out in 4-inch test tubes in a total volume of 0.5 ml. of aqueous solution. The complete system contained 0.05 millimole phosphate (pH 7.0), 0.5 mg. L-ornithine monohydrochloride (Mann Research Laboratories), 1.25 mg. α-ketoglutaric acid (neutralized with sodium carbonate), 2.5 μg. pyridoxal phosphate, 0.5 mg. *o*-aminobenzaldehyde,[26] and enzyme preparation containing about 2 mg. (range, 1–3 mg.) protein. Pyridoxal phosphate was included, because it was found[27] to stimulate the activity of partially purified ornithine δ-transaminase from *N. crassa*. In parallel with each tube containing a complete system, three corresponding control tubes were prepared, from which ornithine, α-ketoglutarate, or enzyme preparation were respectively omitted.

The tubes were incubated at 25° C. for approximately 10 hours and examined for yellow color. Samples giving a positive color test were bioassayed with strain 55-25, according to the method previously described.[22] A growth response dependent on the simultaneous presence of ornithine and α-ketoglutarate in an enzymatic digest was taken to indicate that the enzyme preparation involved has ornithine δ-transaminase activity. The respective enzymes responsible for this activity may or may not be identical in different organisms.[28–30]

The sensitivity of the procedures used is estimated to be such that one-sixth of the lowest transaminase activity found could have been detected on the basis of the color test, and one-third on the basis of the growth response. The following figures will illustrate the range of activities encountered. The mean activity corresponded to about 0.05 μmole L-Δ^1-pyrroline-5-carboxylate formed per tube under the conditions given above. The lowest and highest values found were approximately 10 and 500 per cent of the mean (for *B. megaterium* and *B. subtilis*, respectively; see below). Since crude enzyme preparations were used, the quantitative activity values obtained are perhaps best considered minimum values.

In a survey of an enzymatic activity, it is possible to obtain misleading results, for example, through inadequately specific detection methods ("false positives"); or through insufficiently sensitive assay techniques or unsuitable extraction and preservation procedures ("false negatives"); or (in various conceivable ways) through interfering enzyme activities or inhibitors. With the aid of the methods described, it was endeavored to avoid such misleading results.

RESULTS AND DISCUSSION

The results of the present survey are summarized in Table 1. A clear-cut, consistent distribution pattern of the transaminase activity will be noted. Table 1 shows that ornithine δ-transaminase activity is present in all the gram-positive bacteria, the (gram-positive) yeasts and other fungi, the green algae, the protozoon, and the higher plant and animal tissues examined. In contrast, this transaminase activity could not be detected in any of the gram-negative bacteria or in the (gram-negative[31]) blue-green alga tested. Thus, as judged by the absence of this enzyme activity, the gram-negative bacteria and blue-green algae appear to constitute a group of organisms set apart from other forms of life.

The distribution of ornithine δ-transaminase activity, therefore, is in harmony with available taxonomic information: a relationship between bacteria and blue-

green algae has been inferred, on various grounds, by a number of investigators (cf. Stanier;[32] Pringsheim[31]).

Within the bacteria, the presence or absence of the transaminase activity is seen to correlate with gram-positive (including gram-variable[33]) or gram-negative character, respectively. This correlation is in line with other significant differences between the gram-positive and the gram-negative bacteria (see, for example, Bartholomew and Mittwer;[34] Mitchell and Moyle;[35] Salton[36]).

The presence of ornithine δ-transaminase activity in calf liver supplements the earlier findings of this activity in other mammals.[37, 38] Furthermore, evidence from nutritional or tracer experiments is consistent with the occurrence of this activity in protozoa,[39] mammals,[40] and higher plants.[41]

The results of the present investigation are of special interest in conjunction with the remarkable and comprehensive information available on the occurrence of α,ε-diaminopimelic acid.[42–48] This amino acid gives rise to lysine in *E. coli* by decarboxylation (see review by Work[49]). Since lysine is a homologue of ornithine (which is producible through the transaminase activity now studied), the information on diaminopimelic acid seems particularly relevant.

Investigations of the comparative biochemistry of ornithine and lysine metabolism promise to be rewarding in view of the available results of pathway studies with *N. crassa* and *E. coli*. In *N. crassa* there is evidence that both ornithine and lysine are formed from ω-semialdehydes: ornithine is formed from glutamic γ-semialdehyde,[3] and lysine appears to arise from (the recently obtained)[50] α-aminoadipic δ-semialdehyde.[50–53] In *E. coli*, on the other hand, ornithine and lysine are formed via N-acylated intermediates:[54] for ornithine the acyl group involved is acetyl,[2, 4, 55, 56] and for lysine the acyl group is succinyl.[57] It is thus seen that the homologous amino acids ornithine and lysine are produced through one set of (at least partly) analogous reactions in *N. crassa* and through another in *E. coli*.

Diaminopimelic acid functions not only as an intermediate in lysine synthesis in *E. coli* but also as a characteristic cell-wall constituent in this and other microorganisms.[36, 43, 47, 49] To what extent the two roles of diaminopimelic acid (as lysine precursor and as cell-wall constituent) are associated in various forms of life is an interesting question of comparative biochemistry.

The above-mentioned investigations of the distribution of diaminopimelic acid have revealed a pattern which is consistent with, and complemented by, the pattern of occurrence of ornithine δ-transaminase activity, as manifested in the present results. The two distribution patterns are compared, as far as possible, in Figure 2.

The occurrence of α,ε-diaminopimelic acid, in one or another isomeric form, appears to be confined (with one reported exception;[44, 58] see below) to the bacteria and blue-green algae.[32, 42, 49] Diaminopimelic acid was found in all the bacteria and blue-green algae tested, except in a group of gram-positive bacteria (Group 2 of Fig. 2), including certain cocci[42, 45] and lactobacilli.[45, 46] The gram-positive bacteria in which diaminopimelic acid was found are represented as Group 1 in Figure 2. Diaminopimelic acid could not be detected in any of the fungi, non-blue-green algae, protozoa, or higher forms of life examined,[42, 43] except for a green alga, *Chlorella ellipsoidea*, which seems to contain a relatively low amount of the amino acid.[44, 58]

The distribution studies of ornithine δ-transaminase activity and of diaminopimelic acid are seen to point to two similar, but not identical, dichotomies. As shown in Figure 2, the two points of division split the range of organisms into three parts: except in the (relatively small) central area, the transaminase activity and diaminopimelic acid would seem to be mutually exclusive. Thus the available information indicates that the fungi, non-blue-green algae, protozoa, and higher plants and animals all are similar in possessing the transaminase activity but lacking diaminopimelic acid; in contrast, the gram-negative bacteria and blue-green algae appear to lack the transaminase activity but to contain diaminopimelic acid. The central area in Figure 2 is occupied by a group of gram-positive bacteria (as represented by *B. subtilis*) which have an intermediate position in that they exhibit the transaminase activity *and* contain diaminopimelic acid. A second group of gram-positive bacteria (Group 2, as represented by *M. lysodeikticus*) have affinities to the higher organisms on the basis of both criteria.[59]

In view of the highly suggestive and complementary distribution patterns of ornithine δ-transaminase activity, as revealed by the present study, and of diaminopimelic acid, as discovered by other authors, it might not be unduly optimistic to expect that such surveys will help to shed light on taxonomic, and perhaps even on evolutionary, relationships among micro-organisms and other forms of life.

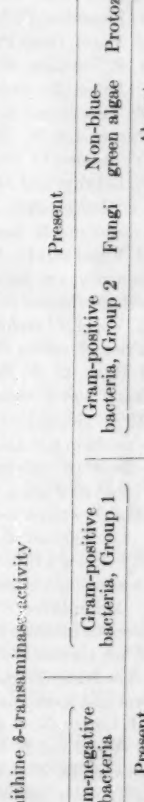

FIG. 2.—Distribution patterns of ornithine δ-transaminase activity and diaminopimelic acid.

SUMMARY

The difference in ornithine synthesis and the concomitant difference in ornithine δ-transaminase activity between *N. crassa* and *E. coli* prompted an investigation of the occurrence of this activity among various organisms.

The transaminase activity was not detected in extracts of any of the gram-negative bacteria or of the blue-green alga tested but was uniformly found in extracts of the gram-positive bacteria, fungi, non-blue-green algae, the protozoon, and the higher plant and animal tissues examined. Thus, in harmony with available information, the group containing the gram-negative bacteria and blue-green algae appears to be set apart from other organisms.

The distribution pattern found is particularly interesting in comparison with the known distribution pattern of α,ε-diaminopimelic acid. The two patterns may well reflect two similar, but not identical, dichotomies in the biological world. A group of certain gram-positive bacteria are intermediate forms in that they occupy a region between the two points of division indicated by the two distribution patterns.

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† The results reported here are taken from a dissertation presented by W. I. Scher, Jr., for the degree of Master of Science in Yale University.

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*ON THE MECHANISM OF TEMPERATURE INDEPENDENCE IN A BIOLOGICAL CLOCK**

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Communicated by E. Newton Harvey, July 14, 1957

Introduction.—Diurnal rhythms which persist under constant conditions have been found in a variety of plants and animals (see Bünning;[1] Pittendrigh[2]). Most of the evidence points to the conclusion that diurnal rhythmicity is endogenous and results from oscillations of physiological processes of one sort or another. An unusual feature of many rhythms, however, is their temperature independence. In instances where study has been directed toward the temperature characteristics (Wahl;[3] Brown and Webb;[4] Pittendrigh;[5] Bruce and Pittendrigh;[6] Bühnemann[7]), it has been found that the period, or duration of a cycle, is unaffected or only slightly affected by temperature changes. The way in which this is achieved in organisms which are otherwise markedly temperature-sensitive is not known. It is possible that some uncontrolled environmental variable might be involved, i.e., that there is an exogenous factor which plays a role in determining the period in rhythmic organisms. Another possibility is that temperature compensation is incorporated as a part of the rhythmic mechanism. In either case, it may be noted that temperature independence constitutes support for the idea that a diurnal rhythm could be related to an accurate biological timing device, or clock. If, in fact, the rhythms are related to a chronometer mechanism, the significance of temperature independence is clear.

We have recently demonstrated (Hastings *et al.*[8]) that the dinoflagellate *Gonyaulax polyedra* possesses a diurnal rhythm of luminescence which persists under conditions of constant low light and constant temperature. Since *Gonyaulax* is a unicellular form which may be grown readily in large quantities and studied biochemically, it is an advantageous material for the study of rhythmicity. In addition, the quantitative measurement of light intensity provides an easy and accurate method for following rhythmic changes. The experiments reported here describe the effect of temperature upon the luminescent rhythm. The results suggest that temperature independence is achieved by means of a compensation mechanism.

Materials and Methods.—*Gonyaulax polyedra* is an armored marine dinoflagellate which is both photosynthetic and luminescent. This organism was isolated from a net sample collected off the pier at Scripps Institution of Oceanography and has been maintained in a unialgal culture in a medium composed of 75 per cent filtered sea water, 2 per cent soil extract, 2×10^{-3} *M* KNO_3, 2×10^{-4} *M* K_2HPO_4, 6×10^{-6} *M* $FeCl_3$, and 2.7×10^{-5} *M* ethylenediamine-tetraacetic acid (EDTA) at pH 8.0–8.2. Under optimal conditions of light and temperature, a growth rate of about one division per day has been measured. Bacteria-free cultures have been found to have a reduced viability and growth rate, apparently due to the lack

of some unknown growth factor which may be supplied by the bacteria. In these experiments, therefore, bacteria-free cultures were not used.

Luminescence in *Gonyaulax* normally occurs only upon stimulation. The cell emits a flash of light having a duration of less than 0.1 second, a record of which has been published previously (Hastings and Sweeney[9]). In our experiments, luminescence was evoked by bubbling a fine stream of purified air through a 2-ml. aliquot of the cell suspension for 1 minute. With continued stimulation, the luminescent response declines sharply, so that after 1 minute, only a very small amount of light is emitted. Due to this fatigue characteristic, the luminescence of a suspension may be quantified by measuring the relatively brief bright burst of light emitted upon stimulation. The light emitted was measured with the photomultiplier apparatus previously described (Sweeney and Hastings[10]), which permits integration of light by accumulating the current output from the phototube on a capacitor. Our index of luminescence therefore is a fixed proportion of the total amount of light emitted by a cell suspension during a 1-minute period of stimulation. After each determination the cell suspension used for luminescence measurement was discarded.

The experiments illustrated in Figure 1 were carried out in the Earhart Laboratory of the California Institute of Technology, where eight different temperature-controlled rooms were available.[11] The temperatures of seven of these rooms and the extreme temperature variations recorded are noted in Figure 1. The eighth room was at 6° C. Unless otherwise stated, the only illumination in these rooms was provided by an overhead fluorescent fixture with two 40-watt "daylight" bulbs placed at a distance of 1 meter from the cultures, where the light intensity was about 100 foot-candles. Uniformity of illumination was achieved by means of a diffusing screen of white tracing paper placed under the fixtures.

Experimental Procedure.—Gonyaulax is an obligate photoautotroph. When cultures are grown with alternating light and dark periods of 12 hours each (= LD), their luminescence is much greater during the dark period than during the light period. If such cultures are then transferred either to total darkness or to continuous dim light (100–200 foot-candles), this rhythm of luminescence persists, retaining in both cases the period of approximately 24 hours. It has been shown (Sweeney and Hastings[10]) that in continuous darkness the amplitude of the rhythm is damped as a result of the depletion of its energy supply. In dim light, however, the luminescent rhythm persists with undiminished amplitude and has been observed for as long as fourteen consecutive days (Hastings *et al.*[8]).

The plan of the experiment was as follows: Two-milliliter aliquots from a culture of *Gonyaulax* which had been growing at 22° C. in LD conditions (light from 6:00 A.M. to 6:00 P.M. at an intensity of 800 foot-candles) were pipetted into each of 1,300 test tubes. The cell density at this time was 3,600 cells per milliliter. The tubes were placed in beakers and returned to the previous LD conditions at 22°. Two days later, just prior to 6:00 A.M., cultures were removed from the dark at 22° and placed in the dark in each of the eight rooms, at different temperatures. A few minutes later, at 6:00 A.M., the lights were turned on in all rooms. The cells therefore were exposed to the transition from dark to light almost simultaneously with the transition from 22° to a second temperature. Thereafter, at

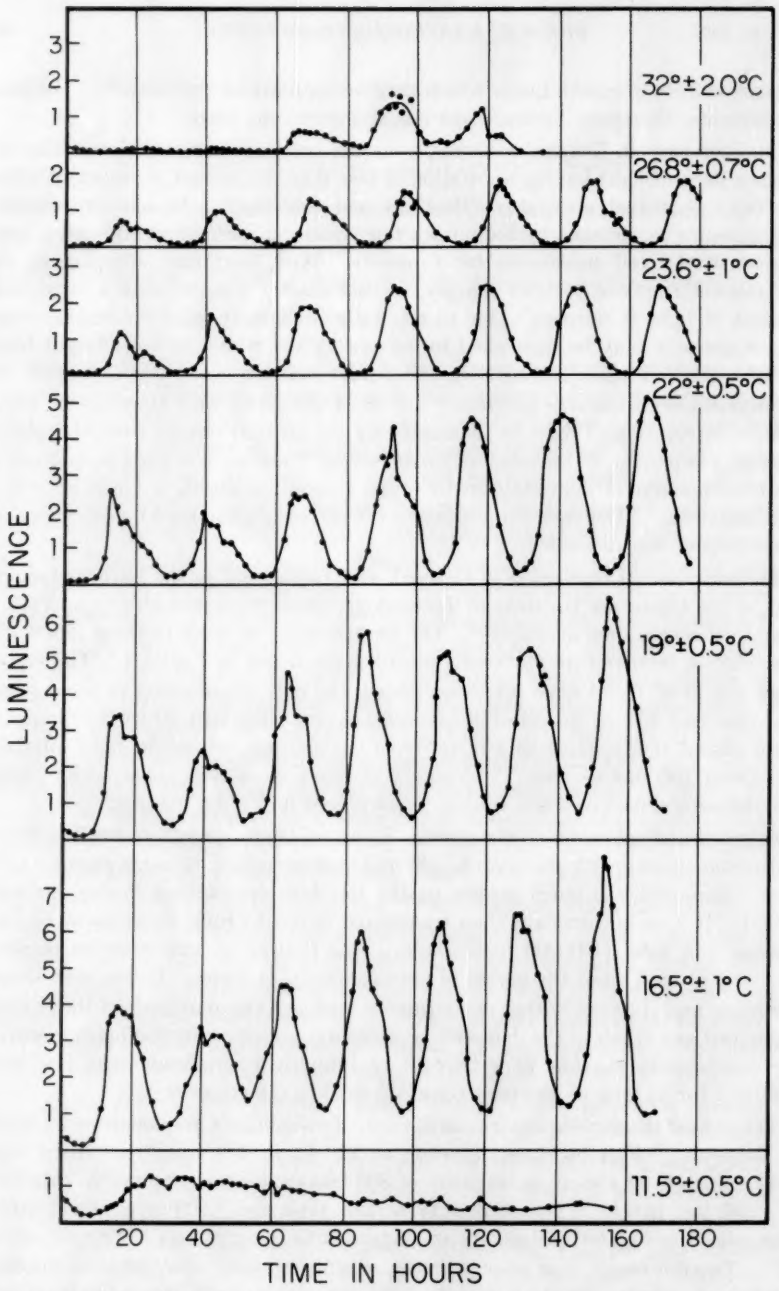

FIG. 1.—Characteristics of the persistent rhythm of luminescence at several different temperatures. The cells were kept at 22° C. in LD conditions prior to the beginning of the experiment shown on the graph. At that time the cells were transferred from the dark at 22° to the various temperatures, all in dim light, and left in these conditions throughout the experiment. The luminescence of the cells was determined approximately every 2 hours. At the dim light intensity used, there is little cell division, even at the optimal temperature for growth. The number of cells therefore remained essentially the same in all tubes at all temperatures.

approximately 2-hour intervals, two tubes were removed from each of the rooms, assayed for luminescence, and then discarded.

Results.—The results of this experiment are shown in Figure 1. At the five temperatures between 16.5° and 26.8° C. the experiments were continued for seven cycles. It is quite evident that the higher the temperature, the longer is the duration of a period. We have estimated the average time of a period at each temperature by taking the time at which the first and last peaks of luminescence occurred and dividing by the number of periods. The data from this computation are plotted in Figure 2.

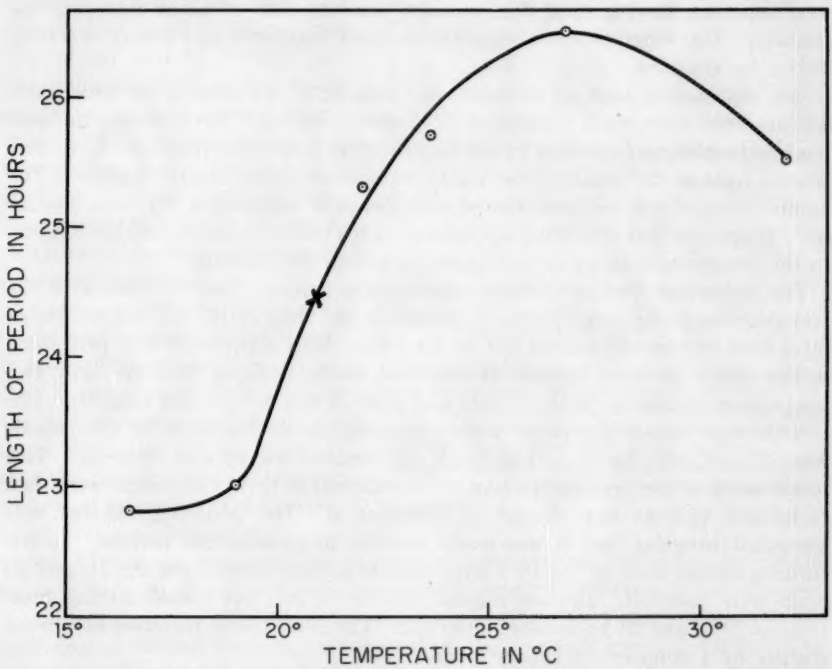

FIG. 2.—The effect of temperature upon the length of a period in the rhythm of luminescence. Data taken from the experiment shown in Fig. 1, except for the point indicated by the symbol ×. This was obtained in an experiment (similar to those shown in Fig. 1) carried out in Evanston several months later at a temperature of 21.0° ± 0.1° C.

The temperature control in the room at 32° C. was poor, and the cells were killed on the sixth day of the experiment, when the temperature rose to 36°. The irregularity of the luminescence is probably due in part to the temperature fluctuations and in part to an adverse effect of the high temperature upon the cells. In spite of this, there is a clearly apparent rhythmicity with a period not far from 24 hours, the average duration being about 25.5 hours.

At a temperature of 11.5° C. the rhythmicity was lost, although the cells retained luminescence and viability. From previous experiments it is known that essentially no growth occurs at 11°. When the cultures at 11.5° were returned to a

temperature of 22°, the rhythmicity was regained, but it was not determined whether or not the rhythmicity would persist for more than one cycle. It has been reported by Brown and Webb[4] that in the fiddler crab, *Uca*, a low-temperature treatment is equivalent to "stopping the clock" and that when the organism is brought back to a higher temperature, the rhythmicity resumes, with the phase displaced by a time period corresponding to the time of low-temperature treatment. This does not occur in *Gonyaulax*. The phase is determined by the time at which the cultures are returned to the higher temperature. Some cultures were removed from the 11.5° room and taken to the 22° room after 58 hours, and others after 67 hours at the lower temperature. In both cases, pronounced rhythms were initiated, having their first maximal luminescence 30 hours following the transfer. This suggests that a temperature shock may serve as a timing or setting device for rhythms.

Cell suspensions kept in constant dim light at 6° C. showed no measurable luminescence, even when warmed to 22° before assaying. Nevertheless, the cells remained viable, as evidenced by the fact that cultures transferred from 6° to continuous light at 22° regained the ability to luminesce after about 12 hours. The luminescence of such cultures showed no evidence of regaining a rhythmic character. It appears that cells which are kept at such a low temperature will not respond to the temperature shock as a stimulus to initiate rhythmicity.

The important finding in these experiments, namely, that the length of the period of the endogenous rhythm is greater at 26° than at 16° C., was confirmed by a later experiment carried out at La Jolla. This experiment was performed in essentially the same manner as described above, utilizing, however, only two temperature rooms (at 15.9° ± 0.2° and 26.6° ± 0.5°). In each case, only two periods were measured; at the higher temperature the durations of the periods were 27 and $26^1/_2$ hours, and at the lower temperature, 23 and 22 hours. This result confirms the previous finding. The cultures in these two rooms were then exchanged without any change in illumination. The persisting rhythm was somewhat irregular, but it was easily possible to measure the periods. In the cultures moved from 26° to 16°, three periods having durations of 20, 21, and 20 hours were observed. In those moved from 16° to 26°, two periods having durations of $24^1/_2$ and 24 hours were observed. The period may therefore be altered slightly by a temperature change alone.

From these experiments it is evident that, although the period does vary with temperature, the effect is not very great. The rates of various processes in chemical and biological systems usually differ by a factor of two or three at temperatures 10° C. apart. In our experiments the length of the period varies by only about 15 per cent at temperatures 10° apart. Since the temperature effect is relatively small, we may speak of this "clock" as being temperature-independent and comparable to the other cases of temperature-independent rhythms which have been reported.

However, the dependence on temperature which we do observe is worthy of consideration. First of all, it essentially eliminates the possibility that the period of the rhythm is regulated directly by some uncontrolled periodic variable in the environment. Second, in view of the fact that the observed Q_{10} is less than 1.0, it definitely suggests that a temperature-compensation mechanism is involved.[12]

If we were to assume that the duration of the period is governed by the rate of a single chemical reaction, then an increase in temperature should result in a period of shorter duration. The effect we observe in this case is precisely the opposite, i.e., the Q_{10} between 16.5° and 26.8° C. is 0.86. This result can be readily explained if we assume that temperature independence is achieved by means of compensation, and that this situation is an example of overcompensation.

There are several other compelling reasons to conclude that temperature independence, in biological clocks generally, results from a temperature-compensating mechanism. The pertinent considerations have been discussed in detail by Bruce and Pittendrigh[6] and by Pittendrigh.[2] For example, the effects upon endogenous rhythms in several organisms of repeated and nonrepeated temperature shocks, and the initiation of rhythms by single temperature shocks, favor the interpretation of a temperature-compensating mechanism.

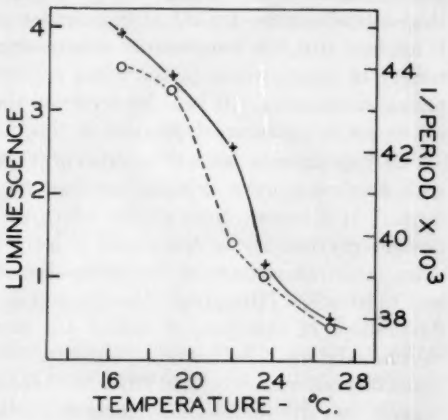

FIG. 3.—Plot showing the effect of temperature upon the amount of luminescence (measured 16 hours after the beginning of the experiment; data plotted with crosses, *solid line*), as compared with the temperature effect upon period (plotted as the inverse of period duration; data plotted with circles, *dotted line*). Data taken from the experiments shown in Fig. 1.

A temperature-compensation model can be expressed very simply in terms of two chemical reactions, A → B and C → D, both of which are temperature-dependent in the usual way. Let it be assumed that the rate of the first reaction controls the duration of the period, while D is an inhibitor of this reaction. If the two reactions have appropriate temperature coefficients, the rate of the first reaction, A → B, will remain essentially constant with temperature changes. If the temperature coefficient of the second reaction was too high in relation to the coefficient of the first reaction, an apparent Q_{10} for A → B of less than 1.0 would be expected. Also, the fact that the average period length in the experiment at 32° C. is less than at 26.8° and 23.6° may be understood in terms of the model reactions by assuming them to be enzymatic and to have different optimal temperatures. The observed result would be expected if the temperature optimum of C → D was somewhat lower than that of A → B.[13]

There is additional evidence for the suggestion that an inhibitor is acting in the luminescent system. In the experiments shown in Figure 1, it can be seen that the intensity of luminescence is less at higher temperatures. In Figure 3, the intensity of luminescence, measured 16 hours after the beginning of the experiment, is plotted against the temperature. On the same graph are plotted the data from Figure 2, using the inverse of time on the ordinate. The parallel between the two implies that both result from the action of the same postulated inhibitor system, even though the intensity of luminescence is changed by a greater degree than is

the periodicity with the temperature change. The unusual relationship between luminescence and temperature has been confirmed in many experiments, the details of which will be published elsewhere. We have found that light emission in the living cell increases with decreasing temperature to as low as 5° C.

In contrast to these findings, we have previously reported[9] that the luminescent reaction in cell-free extracts is temperature-dependent in the usual way. Luminescence increases with increasing temperature to an optimum at 25° C. and decreases thereafter as a result of enzyme denaturation. The growth rate of the cells is also temperature-dependent in a similar way, the optimum being at about 25°. It appears that the temperature relationships of both the periodicity and the intensity of luminescence in the living cell result from some particular and perhaps unique mechanism. It is at least certain that the entire metabolic pattern of the cell is not temperature-dependent in this unusual way.

Our experiments, as well as the previous studies of Bruce and Pittendrigh[6] with *Euglena gracilis*, demonstrate that a clock system may occur in unicellular forms. It is known, from studies which we have reported previously,[9] that luminescent rhythmicity in *Gonyaulax* is associated with diurnal fluctuations in the extractable components of the luminescent system—with respect to both enzyme and substrate. Pittendrigh[2] has pointed out that this is to be expected and that fluctuations of this sort, or indeed any overt and measurable manifestations of rhythmicity, may be merely "outputs," or reflections of the activity of some undefined but master cellular clock mechanism. In contrast to this view, we would suggest that rhythmicity may occur in particular and essentially discrete systems, such as the luminescent system, and that no special clock need be invoked to account for luminescent rhythmicity or, indeed, any other rhythmic process. In support of this idea, we point to the evidence that the temperature-compensation mechanism apparently acts in the luminescent system directly. If there were some other clock to which luminescence was tied, we would expect the clock itself rather than the luminescent system, to be temperature-compensated.

It seems that the general nature of persistent diurnal rhythms must eventually be understood in terms of oscillating chemical systems. On the assumption that discrete biochemical systems may undergo sustained autonomous oscillations, it appears worthwhile to investigate the biochemistry of such systems in detail.

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[12] It is interesting to note that this is the second instance in which a Q_{10} of less than 1.0 has been reported for the length of the period in a rhythmic process. Bühnemann found that the rhythm of sporulation in *Oedogonium* had a period of longer length at higher temperatures.

[13] E. N. Harvey (personal communication) has recently suggested a model based on similar assumptions. We are not of the opinion that the intracellular reactions which are involved in rhythmicity and the temperature-compensation mechanism are as simple as those we have suggested. They are used merely for illustrative purposes. It does not seem worthwhile at the present time to suggest a more elaborate model, in the absence of more specific biochemical information. We would guess, however, that the chemistry of rhythmicity will be shown to involve a heterogeneous system rather than a homogeneous one.

CATALYTIC AND POLYMERIZATION-DIRECTING PROPERTIES OF MINERAL SURFACES

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Recent years have seen an extension of our knowledge of how organic chemical compounds may have been synthesized in preorganismal times. Urey's interpretation of chemical and physical data pertaining to planetary evolution[1] has provided the major stimulus for such work. Miller has demonstrated the ease with which synthesis of amino acids and other organic compounds can be effected in gas mixtures.[2] This paper deals with a possible mechanism of polymerization of such compounds. Polymerization in simple aqueous medium has but a low probability. Bernal[3] has suggested that clays might act as adsorbing catalytic surfaces. In the present work it will be shown that in certain instances rock and mineral surfaces can act as catalytic materials for polymerization reactions.

In earlier work on the synthesis of lignin it was shown that during the enzymatic peroxidation of eugenol, lignin is formed only when the reaction system includes an appropriate matrix such as cellulose, methylcellulose, or pectic acid.[4] It has now been found that several mineral silicates can serve as matrices for the formation of lignin-like products which are, however, different from those formed in the presence of polysaccharides.

METHODS

Exposed surfaces of all rocks and minerals used were removed and specimens treated successively with hot chloroform, ether, hot methanol and ethanol, cold dilute HCl, and boiling water. To provide a check on aromatic contaminants, washings were scanned for ultraviolet-absorbing substances between 220 and 380 mμ (Beckman Model DU spectrophotometer with silica absorption cells). Initial washings sometimes yielded absorbancies as high as 0.05, but later washings were free of such contaminants.

Rocks and minerals were used as individual pieces 1–3 cm.^{3} in volume and 8–10 cm.^{2} in area. Irregular surfaces of fibrous minerals were greater in area but of the

same order of magnitude, and the extensive internal surface of the pumice used was not measured.

Procedures for analysis of reaction products included (*a*) solvent fractionation followed by ultraviolet absorption measurements and color tests (HCl-phloroglucinol) for lignin-like polymers; (*b*) NaCl precipitation followed by colorimetry (Bausch and Lomb Spectronic 20 spectrophotometer), ultraviolet absorption measurements, and C and H analysis for catechol and phenylalanine polymers; (*c*) iodimetric titration for determination of H_2O_2; and (*d*) colorimetric determination of inorganic phosphates released from glucose-1-phosphate with ammonium molybdate.

Experiments involving moderate pressures were executed in a steel-jacketed, glass-lined corrosion test pressure bomb.

EXPERIMENTAL RESULTS

Oxidative Polymerization of Eugenol.—In the previous work on lignin formation, the enzyme peroxidase was used to catalyze the peroxidation of eugenol. In the present experiments, combined heat and pressure replace the enzyme.

In a representative case, three reaction mixtures, each containing eugenol and water, were maintained at 128° C., 5 atmospheres pressure, for 3 hours. One tube contained no mineral, a second tube contained 70 mg. of chrysotile (serpentine), and in the third, 50 mg. of chrysotile was suspended in the pressure bomb. After pressure reaction, the water-insoluble products were isolated and analyzed.

A eugenol polymer will be considered lignin-like if it is insoluble in chloroform and ethanol; soluble in dioxane, ethanol–1 per cent HCl, or both, gives a red-to-magenta color with phloroglucinol-HCl, and exhibits an ultraviolet absorption peak in the 275–280 mμ range or in the region of 315 mμ. The results (Table 1) show that the

TABLE 1*

EFFECT OF CHRYSOTILE ON THE POLYMERIZATION OF EUGENOL WITH HEAT AND PRESSURE

	Control Tube			Chrysotile Tube			Chrysotile in Vapor Phase		
Fraction Soluble in	Per Cent	Abs. Max. (Mμ)	Color Test	Per Cent	Abs. Max. (Mμ)	Color Test	Per Cent	Abs. Max. (Mμ)	Color Test
Chloroform	42	280	+	5	280	+	8	280–285	+
Ethanol	8	280	+	18	280	−	41	280	−
Dioxane	0	None	−	0	None	−	41	None	−
Ethanol–1 per cent HCl	12	None	−	77	315	+++	10	315	+?
None	38	None	−	0	None	−	0	None	−
Total yield (mg.)	..	26.7	...	..	25.9	...	..	60.4	...

* Each tube contained 1 mmole eugenol plus 100 mmoles H_2O_2; one tube contained no mineral, one tube contained 70 mg. chrysotile, and in the third tube 50 mg. chrysotile was suspended in the vapor phase. Reaction carried out at 128° C., 5 atmospheres, for 3 hours.

presence of chrysotile alters the array of products formed from eugenol and water during incubation at 128° C. and 5 atmospheres pressure. A lignin-like polymer is indeed formed when the mineral is present. This material is not formed in the absence of chrysotile.

In other experiments eugenol and hydrogen peroxide were allowed to react at 25° C., 1 atmosphere pressure, in the presence of various minerals. Polymer formation in such cases assumed a pattern different from that described above. After a reaction time of 100 hours, some unused eugenol remained. Yields of

polymer were small, but dioxane-soluble fractions were conspicuous in amount. The minerals used as catalysts, present in the reaction mixtures as single pieces weighing 400–500 mg., were extracted repeatedly with hot chloroform and ethanol until all soluble material with absorption in the 260–290 mμ region had been removed. Dioxane-soluble products were then extracted and examined for their lignin-like properties (ultraviolet absorption, color reactions). The data of Table 2

TABLE 2*

EFFECT OF VARIOUS MINERALS ON FORMATION OF A DIOXANE-SOLUBLE EUGENOL POLYMER

MINERAL	ABSORBANCY AT 280 Mμ MAXIMUM	PHLOROGLUCINOL COLOR	MINERAL	ABSORBANCY AT 280 Mμ MAXIMUM	PHLOROGLUCINOL-COLOR
Chrysotile (serpentine)	0.462	Pink	Pumice	>3	Magenta
Granite	0.880	Yellow	Quartz	0.005	Yellow
Garnet	0.170	Yellow	Selenite	0.107	Yellow
Hornblende (amphibole)	2.900	Pink	Calcite	0.015	Yellow
Muscovite	>3	Deep pink	None	0.085	None
Biotite	0.650	Yellow			

* 1 mmole of eugenol and 10 mmole H_2O_2 were incubated in 10 ml. H_2O with various mineral solids (as intact pieces of 400–500-mg. weight) for 100 hours at 25° C., 1 atmosphere. Chloroform- and ethanol-soluble fractions were removed and the dioxane-soluble fraction tested for lignin. All dioxane-soluble fractions possessed absorption maxima at 280 mμ.

show that calcite and quartz fail to catalyze the production of lignin-like material from eugenol. Pumice and muscovite, on the other hand, are highly active. Chrysotile, hornblende, muscovite, and pumice are similarly able to catalyze the production of lignin-like material from eugenol. The dioxane-soluble materials formed from eugenol in the presence of pumice yield, on analysis, C 60.5 per cent, H 6.1 per cent, in agreement with the composition of natural and synthetic lignins.

Oxidative Polymerization of Other Aromatic Compounds.—Several minerals catalyze the peroxidation of catechol to melanin-like products. Reaction mixtures were incubated at 25° C. for 75 hours, with periodic determination (at 425 mμ) of pigment development. Development of color in controls lacking mineral was light, their absorbancy remaining at less than 0.2 after 75 hours. Absorbancy of reaction mixtures containing biotite, quartz, or pumice was equal to or slightly less than that of the control mixtures. Reaction mixtures containing other minerals, however, formed dark brown materials which could be precipitated by saturation of the system with NaCl at 50° C. (Table 3). Such precipitates could not be re-

TABLE 3*

NACL-PRECIPITABLE CATECHOL DERIVATIVES FORMED IN THE PRESENCE OF MINERALS

MINERAL	POLYMER YIELD (1 PER CENT OF INITIAL CATECHOL)	ABS. MAX. (Mμ)	$E^{1\%}_{1\ cm.}$	ANALYSIS PER CENT C	H
Chrysotile	27	Shoulder 265–270	125	54.0	3.3
Granite	23	265	103	49.8	3.0
Hornblende	32	Shoulder 260–265	411	47.2	3.0
None	3	Shoulder 275–280	260	60.2	3.6

* 1 mmole of catechol and 10 mmoles of H_2O_2 in 10 ml. water were incubated 75 hours at 25° C. with 850–1,000 mg. of material. The NaCl-precipitable material was isolated and analyzed. Spectral determinations were carried out in solutions of this material made in ethanol–1 per cent HCl.

dissolved in water, methanol, ethanol, chloroform, dioxane, or other neutral solvents. They were, however, slowly soluble in ethanol–1 per cent HCl, and spectral determinations were made upon solutions so prepared. The catechol polymers formed in the presence of different minerals differed in yield, in elementary analyses

and in spectral properties, as summarized in Table 3. Reaction mixtures in which *dl*-phenylalanine hydrochloride was substituted for catechol developed brown precipitates in 70–90 hours at 60° C. Chrysotile, garnet, and granite were the most active catalysts for this reaction, based on rate of soluble pigment formation measured at 425 mμ. Reaction mixtures with selenite or quartz, or control reaction mixtures containing no added mineral, formed no such precipitates and exhibited only a trace of yellow color. Material formed on chrysotile yielded the composition C 48.2 per cent, H 3.8 per cent. This is to be compared with phenylalanine: C 65.4 per cent, H 6.7 per cent. The benzene ring of phenylalanine is apparently oxidized in the presence of chrysotile, suggesting that the mineral catalyzes the homolytic cleavage of H_2O_2 to yield OH radicals which then attack the aromatic ring.

Catalytic Decomposition of Peroxide.—During the course of the experiment described above, it was noted that the oxidation rate curves in the presence of the several minerals, chrysotile in particular, attain asymptotic values abruptly. This suggests an unexpectedly rapid depletion of reactants. In order to discover whether the presence of minerals hastens H_2O_2 decomposition, reaction mixtures of H_2O_2 and various minerals were incubated at 25° C. and residual peroxide determined periodically. Chrysotile and other minerals do, in fact, catalyze the decomposition of peroxide (Table 4). Calcite, graphite, and quartz also possess this ability to some degree. The kinetics of the decomposition process may be first order, as is the case with chrysotile (and in the case of catalyase), or second order, as is the case with hornblende.

TABLE 4*

DECOMPOSITION OF H_2O_2 BY MINERALS

MINERAL	PER CENT DESTRUCTION AFTER 36 HOURS	ORDER OF REACTION	MINERAL	PER CENT DESTRUCTION AFTER 36 HOURS	ORDER OF REACTION
Granite	27.1	1st	Muscovite	9.7	2d
Graphite	12.5	2d	Garnet	15.7	2d
Kaolin	12.5	2d	Chrysotile	50.0	1st
Calcite	11.6	2d	Pumice	13.2	2d
Hornblende	25.7	2d	Quartz	7.9	2d
Selenite	23.8	2d	None	<1	2d

* To vessels containing 10 mmoles of H_2O_2 in 20 ml. H_2O were added 700–800-mg. pieces of various minerals, and H_2O_2 decomposition followed iodimetrically for 36 hours at 25° C. Vessels were incubated in darkness. Order of reaction was determined from rate curves.

Hydrolysis of Glucose-1-phosphate.—Mineral surfaces appear to be able to catalyze still further types of reactions. Thus certain minerals catalyze the hydrolysis of glucose-1-phosphate. Glucose-1-phosphate (6 μmoles/ml.) was incubated with 550–600-mg. samples of mineral for 140 hours at 75° C. Free phosphate was determined colorimetrically during the course of the incubation. Control reaction mixtures containing no mineral yielded 10 ± 2 per cent hydrolysis of glucose-1-phosphate during the experimental period. In the presence of chrysotile, hydrolysis was increased to 25 ± 1 per cent. Other rocks and minerals, including calcite and garnet, as well as granite, were unable to catalyze this hydrolysis.

The hydrolysis which takes place in the control reaction mixtures would appear also to be in part catalytic and, in fact, catalyzed by the glass walls of the reaction

vessel. Thus reaction mixtures in which Teflon vessels were substituted for glass exhibited virtually no hydrolysis (<1 per cent) during the entire reaction time.

DISCUSSION

The above experiments indicate that mineral surfaces not only are capable of catalyzing the formation of lignin-like polymers but also exhibit varied further catalytic properties. No single mineral among those tested above is pre-eminently superior to all others in all activities, although chrysotile possesses a high level of versatility. It would appear unlikely that the catalytic activities are due solely to the presence of iron or other metal ions, since, if this were the case, it would be expected that a single order of decreasing catalytic effectiveness would hold true for all types of reactions. This is not the case, as indicated by the data of Tables 2, 3, and 4. Individual minerals exhibit a sort of substrate specificity. Whether such selectivity is applicable to all samples of the same mineral is not known.

It is proposed that the present data may be considered as evidence for product specificity of mineral catalysts. The first-formed product of a catalytic process, often a free radical, would appear to be able to react in different ways according to the crystal structure and composition of the individual mineral. It will obviously be of interest to extend these observations to possible catalytic participation of mineral surfaces in the polymerization of amino acids and nucleotides as well as to other types of chemical reactions.

SUMMARY

Eugenol, catechol, and phenylalanine were subjected to heat and pressure or exposed to H_2O_2 in the presence of various rocks and minerals, including chrysotile, granite, micas, calcite, and others. In the presence of chrysotile, eugenol-water mixtures form an array of products, including lignin-like polymers, at 128° C., 5 atmospheres pressure. These products are different from those formed in the absence of mineral. At 25° C. the three phenols form different products on different mineral surfaces. These brown salt-precipitable catechol derivatives vary in spectral properties and in elementary composition according to the mineral upon which they are found. Phenylalanine is oxidized to a brown insoluble product in the presence of an appropriate mineral. This oxidation, as catalyzed by chrysotile, appears to involve extensive hydroxylation. Mineral surfaces also catalyze the decomposition of H_2O_2 and the hydrolysis of glucose-1-phosphate.

The mildness of the conditions used, as well as the use of natural minerals as catalysts, make the present work relevant to problems of chemical transformation under early terrestrial conditions and provide an experimental approach to biopolymer genesis in the prebiological stage of evolution.

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*ON THE ORIGIN OF POTENTIAL BARRIERS TO INTERNAL ROTATION IN MOLECULES**

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It is well known that in molecules such as ethane (CH_3—CH_3) one part of the molecule can rotate relative to the other part about the single bond (here C—C) joining the two halves. This phenomenon has considerable importance; for instance, it permits the coiling and uncoiling of protein molecules and other polymers. It has also been known[1] for some time that this internal rotation is not free but is hindered by a potential energy barrier of a magnitude beyond theoretical expectations. Various hypotheses have been put forward concerning the origin of these forces, but no satisfactory conclusion has been reached.

Recent developments in microwave spectroscopy and the related theory[2–10] have provided powerful new tools for the study of this phenomenon. For a certain class of molecules it is now possible to obtain values of the potential barrier with an accuracy of 5 per cent or better, and with much greater certainty than was associated with older methods. Further, the structure and interatomic distances can be obtained and the equilibrium orientation of the rotating groups, as well as other detailed information mentioned below.

Current theories of the forces between atoms suggest a number of types of interaction which could possibly account for the observed barriers. All these forces are of course fundamentally electrostatic interactions among the electrons and nuclei involved. Eyring[11,12] and collaborators early made a quite detailed attempt to find the origin of the barriers by means of the quantum-mechanical approximations then available, but without success. Recently Mason and Kreevoy[13] and also van Dranen[14] have made new estimates of the importance of the van der Waals repulsion between the attached groups, a repulsion which appears between separate gas molecules at close distances, due to overlap of the charge clouds and quantum-mechanical exchange. At somewhat longer distances than occur in most examples of hindered rotation, this repulsion should be replaced by a weak attraction due to inductive and dispersion effects.

The separate atoms and chemical bonds in a molecule will surely interact, due to the direct electrostatic force between the charge distributions, even if there is no important contribution from overlap, exchange, dispersion, or induction. If the electron distribution were known, this term could be calculated with purely classical

methods. This effect has been invoked and investigated by Lassettre and Dean,[15] Oosterhoff,[16] and Au-Chin Tang.[17]

Finally, it has several times been suggested[18] that the potential might not appreciably involve direct interactions of the attached atoms or their bonding electrons at all but might be in some way an intrinsic lack of cylindrical symmetry in the axial chemical bond itself.

Recent microwave data throw some light on the relative importance of these forces. Table 1 shows some barrier values and equilibrium orientations, all but

TABLE 1

SOME POTENTIAL BARRIERS AND EQUILIBRIUM ORIENTATIONS

Molecule	V (Kcal/Mole)	Orientation	Ref.
CH_3CH_3	(2.7–3.0)	(Staggered)	1
CH_3CH_2F	3.30		2
CH_3CHF_2	3.18		2
CH_3CH_2Cl	....	Staggered	3
CH_3OH	1.07		4
CH_3SH	0.71		5
CH_3CHO	1.15	H—H staggered	6
CH_3CFO	1.08	H—F staggered	7
CH_3CClO	1.35	H—Cl staggered	8
CH_3COCN	1.27		9
CH_3NH_2	1.90		10
CH_3SiH_3	1.70	Staggered	11
CH_3SiH_2F	1.56	Staggered	12
CH_3SiHF_2	1.32		13
CH_3CHOCH_2	2.56		14
CH_3CHCH_2	1.98		15
CH_3CHCCH_2	1.59		15
CH_3CHCHF (*trans*)	2.15		16
CH_3COOH	0.48		17
CH_3GeH_3	>2.5		18
CH_3NO_2	0.006	6-fold	19
CH_3BF_2	0.014	6-fold	20

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ethane from microwave frequency measurements. First of all, the equilibrium orientations so far well established (some by electron diffraction[19] as well as spectroscopic methods) show that the forces act like repulsions between the attached atoms. Second, it is notable that in methyl silane and in the aldehyde group of acetaldehyde the replacement of hydrogen by fluorine lowers the barrier. If van der Waals repulsion were the dominant force, this would seem very surprising. In the ethane derivatives, the first fluorine increases the barrier, but not greatly, and the second lowers it. Even the substitution of chlorine for the aldehyde hydrogen in acetaldehyde raises the barrier by only a small amount, and the somewhat uncertain data for the ethane series do not suggest a large increase, as long as one end remains a methyl group. Furthermore, the current picture of van der Waals forces calls for them to decrease very rapidly with distance. Consequently, even if the whole barrier in ethane were due to this cause, simple calculations[20] show that only a fraction of the observed barrier in methyl silane could come from this source. The equilibrium orientation of acetaldehyde has a methyl hydrogen opposite the oxygen and therefore as far as possible from the aldehyde hydrogen. Even granting the different distances, one has difficulty understanding this in terms of van der Waals forces. In view of this whole picture, it seems unlikely that the van der Waals basis is acceptable, although when there are large atoms on both ends or specially short distances, it would be expected to play a role.

The work of Lassettre and Dean[15] reopened the electrostatic-interaction hypothesis by postulating large quadrupole moments in C—H bonds. They expanded the effect of the charge distribution in multipoles, about the midpoint of the bond, including, however, only the dipole and quadrupole terms. Lacking empirical data on the quadrupole moments, they adjusted these to fit the observed barriers and obtained values which have been criticized by some as being unreasonably large. However, the main difficulty was suggested by Lassettre and Dean themselves and is that the multipole expansion as they used it is almost certainly an inadequate approximation. Oosterhoff[16] modified this method by directly calculating the Coulomb interaction of the protons and the spherical electron clouds shielding the protons, reserving the multipole expansion for that part of the electron distribution not expected (on simple quantum-mechanical grounds) to be spherically distributed about either carbon or hydrogen, i.e., the overlap charge between hydrogen and carbon. Further, he included octupole terms. It is therefore much more likely that his expansion was convergent. His treatment was based on a particular quantum-mechanical approximation and gave the result that the observed barrier in ethane could be accounted for, but only if the C—H bond were surprisingly ionic, with a much larger bond dipole moment than is currently supposed.

It is possible to make simple Coulomb calculations of electrostatic interactions based on charge distributions consisting of spheres of charge (with any radial distribution as long as overlap between bonds is negligible) about the hydrogen, the central atom, and any desired point in the bond. Such distributions presumably cannot reproduce accurately the true electron distribution, but they have considerable flexibility, and it would be surprising if they did not permit at least a rough representation of the actual situation. For both ethane and methyl silane they show that the observed barriers can be obtained only by very ionic distributions. For example, in CH_3SiH_3 the maximum possible electrostatic barrier, that which

would arise from bare protons, i.e., a completely ionic state, is calculated to be only 2.8 Kcal., compared with the observed value of 1.7. In methyl germane (CH_3GeH_3) the bare proton calculation gives 2.5 Kcal. Although no experimental value is available for the barrier in this molecule, a preliminary analysis[21] of the microwave spectrum appears to exclude any value below about 2.5 Kcal. These results rule out the electrostatic mechanism as the primary cause in such molecules.

In addition to the above arguments, there is the fact that substitution of the acetyl hydrogen by fluorine does not reverse the equilibrium orientation found in acetaldehyde, despite the general belief that fluorine is more electronegative than oxygen.

There exists another source of information which is in principle more searching than any of the above, partly because it avoids the necessity for comparing different molecules. Rotational transitions are observed in the microwave region not only for molecules in the ground vibrational state but also for those in the lower excited energy levels of vibration and especially internal torsion. These latter, particularly, are found to be well displaced from the ground-state lines, presumably because of the distortion of the molecule produced during the torsional motion by the forces responsible for the barrier. For example, if direct repulsions between the hydrogens were important, the hydrogens should be pushed back as the internal torsion brings them opposite one another. This effect has been treated in a very general way by Kivelson,[4] and his formulas can be applied to several molecules. If a sufficient number of isotopic species are measured, it is possible to obtain values of the derivatives of the hindering potential with respect to the various molecular coordinates. For example, one can solve for the effect on the barrier of pushing the hydrogens further apart. Hecht and Dennison[22] have given approximate results for methyl alcohol. I have carried out such calculations for methyl silane,[4] methyl mercaptan,[23] and methyl alcohol.[22] With the reasonable assumption that there is little effect of stretching the X—H bonds (X = C, O, or Si) and that the vibrational force constant matrix is approximately diagonal, the result obtained is that moving the hydrogens has less effect on the barrier than changing the length of the axial bond.

Unfortunately, the geometrical factors are such as to make the calculations somewhat insensitive to the displacement of the hydrogen atoms; furthermore, only estimates are available for the force constants. These results are therefore not completely conclusive, but they nevertheless provide an important argument against any theory involving direct forces between the hydrogens or bonding electrons to the hydrogens. Calculations based on an inverse power of the distance between hydrogens (or between midpoints of bonds) give an effect of bending the X—H bonds which is somewhat bigger than the effect of stretching the axial bond, whether the power used is 1 (Coulomb law), 4, or 10 (van der Waals), and these are therefore all in conflict with the experimental results.

It should also be mentioned that Linnett and collaborators,[24] from a study of the vibrational potential functions for water and for methane, found that direct forces of repulsion between the hydrogens seemed to be unimportant.

The conclusion to which all these arguments lead is that potential barriers to internal rotation, at least in the case where one group carries only hydrogen atoms, must in some way be an inherent property of the axial bond itself and not due in

any substantial measure to direct forces between the attached atoms or those parts of the electron distribution which are out on the attached bond any considerable distance.

A possible way of obtaining confirmation of this conclusion is to measure barriers in various excited vibrational states. The larger amplitudes of vibration should lead to changes in barrier that are approximately predictable on the basis of the various hypotheses, since the average of the square of the vibrational amplitude will be important. Thus excitation of stretching of the axial bond should have a relatively larger effect than excitation of the bending modes, if the above conclusion is correct. Such observations would be difficult but are probably possible.

This conclusion that it is the electron distribution in and near the axial bond which must play a major role suggests that the quantum-mechanical description of this situation should now be re-examined.

I should like to acknowledge the importance of the discussions on this problem which I have had with Dr. Thérèse Wilson and with Dr. R. W. Kilb, Dr. C. C. Lin, Dr. W. J. Tabor, Mr. Dudley Herschbach, Mr. Victor Laurie, and others who have worked with me on the determination of barriers by the microwave method.

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† Senior Postdoctoral Fellow, National Science Foundation, spring, 1957, Geneva, Switzerland.

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*SOME ASPECTS OF RIBONUCLEIC ACID SYNTHESIS IN ISOLATED CELL NUCLEI**

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In previous experiments it was observed that cell nuclei isolated from calf thymocytes are capable of incorporating glycine-1-C^{14} into nucleic acid purines and that orotic acid (labeled with C^{14}) is an effective precursor of ribonucleic acid pyrimidines in this system. It was also found that the ribonucleic acids (RNA) of the nucleus could be fractionated to yield nucleic acids with different specific activities and diverse time courses of orotic acid–6-C^{14} incorporation[1, 2]. Other workers have recently described further experiments on the metabolic "heterogeneity" of ribonucleic acid in the thymus nucleus.[3, 4]

It is the purpose of the present report to describe a method of fractionation of nuclear ribonucleic acids and its application to tracer studies of RNA synthesis in the isolated nucleus. The results offer a convincing illustration of the complexity of nuclear RNA and demonstrate the separate and widely divergent metabolic activities of two different ribonucleic acid fractions.

Nuclei were isolated from calf thymus tissue in 0.25 *M* sucrose–0.003 *M* $CaCl_2$ solution and suspended in a buffered sucrose medium supplemented with sodium ions, as previously described.[2] Nuclear suspensions were incubated aerobically at 38° C. in the presence of orotic acid–6-C^{14} or adenosine-8-C^{14}. Samples were withdrawn at 10-minute intervals. The uptake of radioisotope was halted by the immediate addition of 0.1 volume of 0.05 *M* sodium cyanide solution (dissolved in the buffered sucrose incubation medium). The nuclear suspension was then centrifuged at 3,000 *g* for 5 minutes, and the supernate was discarded.

The sedimented nuclei contain 1.0 per cent ribonucleic acid. For measurements of the specific activity of this "total RNA," 30-mg. portions of the nuclear sediment were washed four times in the centrifuge with cold 2 per cent $HClO_4$, twice with a 3:1 ethanol-ether mixture, and once with ether. The nuclei were then suspended in acetone and plated on filter-paper planchets, as previously described.[2] Radioactivity was measured using a gas-flow G-M tube with a "Micromil" window of density less than 150 μg/cm.[2] Counts were subsequently corrected for self-absorption. From the known RNA content of the treated nuclei, the over-all specific activity of the total RNA could be calculated. Since the DNA is not removed in this simple procedure, the validity of the calculation depends on the fact that very few of the counts taken up represent DNA synthesis. This is best demonstrated by experiments in which the nuclei, after counting, were treated with ribonuclease and counted again (Table 1). It can be seen that more than 85 per cent of the incorporated adenosine-8-C^{14}, and more than 93 per cent of the incorporated orotic acid–C^{14}, can be removed from the nuclei by RNAase treatment.

The procedure used for the fractionation of the nuclear ribonucleic acids resembles that previously applied to the separation of thymus nuclear proteins.[2] The nuclei, after centrifugation (300–400 mg. dry weight, about 1.5–2.0 gm. wet weight), were taken up in 10 ml. of cold 0.1 *M* potassium phosphate buffer at pH 7.1. (All subse-

quent steps were performed at 2° C.) The resulting gel was homogenized in a small stainless-steel blender for 30 seconds, and the homogenate was centrifuged at 3,000 *g* for 10 minutes. The supernate, which contains the "pH 7.1–soluble RNA fraction," was carefully removed. Trichloroacetic acid was added to it, to bring the final TCA concentration to 10 per cent. The resulting precipitate was washed four times with 2 per cent $HClO_4$, twice with a 3:1 ethanol-ether mixture, and once with ether. It was suspended in acetone and plated on filter-paper planchets for counting. After measurement of radioactivity, the RNA content of this pH 7.1 fraction was determined, using Webb's procedure,[5] and the specific activity of the RNA was calculated accordingly.

TABLE 1

EFFECT OF RIBONUCLEASE UPON RADIOACTIVITY INCORPORATED INTO ISOLATED THYMUS NUCLEI

ISOTOPE INCORPORATED	TOTAL RADIOACTIVITY*		ACTIVITY REMOVED (PER CENT)
	Before RNAse	After RNAase	
Orotic acid–6-C^{14}	3,490	160	95
	3,280	165	95
	3,240	166	95
	3,800	126	97
	3,310	218	93
Adenosine-8-C^{14}	3,365	450	87
	3,620	395	89
	3,680	486	87
	3,211	359	89

*Counts per minute per 30 mg. (dry weight) of nuclei.

The gelatinous residue which remains after extraction with pH 7.1 phosphate buffer was washed with 0.9 per cent NaCl solution and then taken up in 70 ml. of 1 *M* NaCl. After a prolonged extraction with vigorous stirring, a viscous, opalescent solution was obtained, which was then centrifuged at 60,000 *g* for 2 hours. The clear supernate, which contains the DNA, was decanted and discarded. The sediment was washed with cold 2 per cent $HClO_4$, alcohol-ether, and ether, as described above. After measuring its radioactivity, the RNA content of this "1 *M* NaCl residue" was determined, and the specific activity of the RNA was calculated. It will be seen, in what follows below, that this fraction contains ribonucleic acid of intense activity; but, apart from this, special biological interest attaches to this fraction. This interest stems from the cytochemical demonstration by Bessis[6] that an extraction with strong salt solutions at neutrality removes the DNA and leaves the nucleolus with much of its ribonucleoprotein intact. This is evidence that the ribonucleic acid found in the "1 *M* NaCl residue" contains RNA of the nucleolus. It should be emphasized, however, that the presence in this fraction of other, non-nucleolar RNA's cannot be precluded and also that some of the material of the nucleolus may have been removed.

Calf thymus nuclei, as isolated, contain about 1.4 per cent ribonucleic acid. Some of this RNA is readily lost into the incubation medium, and after 30 minutes of incubation, the nuclei contain 1.0 per cent RNA. The pH 7.1 fraction, prepared as described, comprises 4.2–6.0 per cent of the dry weight of the nucleus and contains 8.4–8.6 per cent RNA. The "1 *M* NaCl residue" comprises 2.3–3.5 per cent of the nucleus and contains 8.5–10.9 per cent RNA. Thus the amount of RNA in the pH 7.1 fraction is about 0.4–0.5 per cent of the nuclear mass and greatly exceeds

the RNA of the 1 *M* NaCl residue, which is only 0.25–0.3 per cent of the nucleus. The base compositions of these two different RNA fractions are listed in Table 2.

TABLE 2

PURINE, PYRIMIDINE NUCLEOTIDE COMPOSITION OF RIBONUCLEIC ACID FRACTIONS PREPARED FROM CALF THYMUS NUCLEI

RNA Fraction	Molar Ratios (Relative to Adenylic Acid as 10)			
	Adenylic Acid	Guanylic	Cytidylic	Uridylic
pH 7.1–soluble RNA	10	16.7	16.3	13.1
"Nucleolar" RNA	10	16.7	15.2	12.9

Analyses were performed after alkaline hydrolysis of the isolated fractions by ionophoresis of the nucleotides on filter paper.[7] It can be seen that whatever the differences in base composition among these RNA preparations, the differences are small.

Striking differences are observed in the pattern of orotic acid and adenosine incorporation into the ribonucleic acids of these two fractions. Figure 1 shows the time course of uptake of orotic acid–6-C^{14} into the "pH 7.1–soluble RNA" and the RNA of the 1 *M* NaCl residue. It can be seen that both isolated RNA fractions are metabolically active and that uptake begins without any indication of the delay or "lag period" which characterizes similar experiments on amino acid incorporation.[2] The RNA of the 1 *M* NaCl residue is exceedingly active—about ten times more active than the nucleic acid extracted in 0.1 *M* phosphate buffer. A second difference appears in the time courses of C^{14} incorporation. Orotic acid uptake into the more active fraction proceeds almost linearly for 30 minutes. On the other hand incorporation into the "pH 7.1–soluble RNA" tapers off after 10 minutes. A further difference in metabolic behavior was demonstrated in experiments in which DRB (5,6-dichloro-β-D-ribofuranosyl-benzimidazole) was employed to inhibit RNA synthesis.[2] In these experiments the inhibitor was added to nuclear suspensions in amounts sufficient to reduce over-all orotic acid incorporation by 50 per cent (0.1 mg. DRB per 30 mg. of nuclei). After incubation for 30 and 60 minutes, the RNA's were fractionated as described above. The specific activity of the RNA's in fractions prepared from "control" nuclei were compared with the activities of the corresponding RNA's from "DRB-treated" nuclei. The results are summarized in Table 3. It can be seen that the RNA's in these different fractions differ greatly in their susceptibility to DRB inhibition.

The time courses of adenosine-8-C^{14} incorporation into nuclear RNA's parallel those obtained using orotic acid as a precursor (Fig. 2). Again the RNA of the 1 *M* NaCl residue is ten times more active than the nucleic acid extracted in the 0.1 *M* phosphate buffer, and the time courses of incorporation show the same contrast in duration of uptake.

TABLE 3

EFFECT OF DRB ON OROTIC ACID–6-C^{14} UPTAKE INTO TWO FRACTIONS OF NUCLEAR RNA

RNA Fraction	Time (Min.)	Specific Activity of RNA		Inhibition (Per Cent)
		In "Control" Nuclei (CPM/Mg.)	In "DRB" Nuclei (CPM/Mg.)	
pH 7.1–soluble RNA	30	1,160	702	39
	60	1,290	868	33
"Nucleolar" RNA	30	9,530	3,830	60
	60	14,140	6,490	54

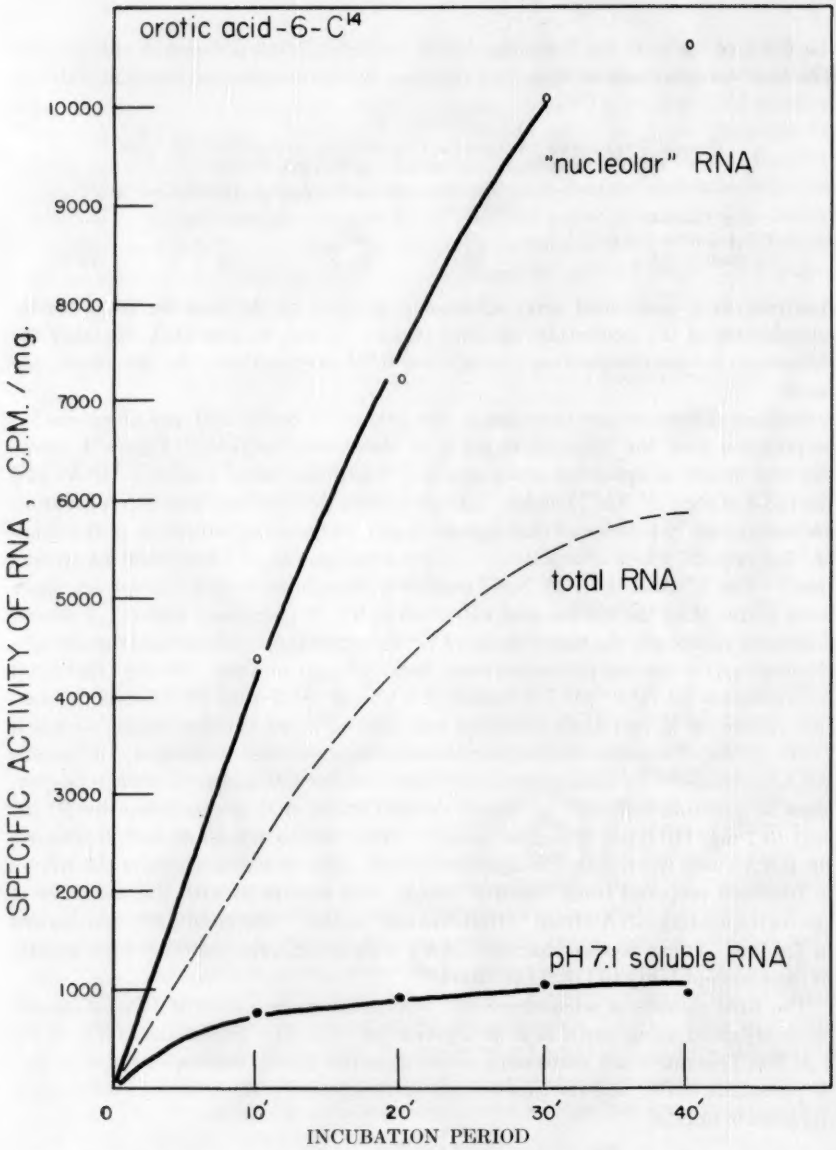

FIG. 1.—The time course of orotic acid-6-C^{14} incorporation into the total RNA and into different RNA fractions of isolated thymus nuclei. The specific activity of the RNA is plotted against the time of incubation. The incubation medium (see Allfrey, Mirsky, and Osawa, *J. Gen. Physiol.*, **40**, 451, 1957) contained 0.05 mg. of orotic acid (specific activity, 1.0 mc/mM) per 40 mg. of nuclei.

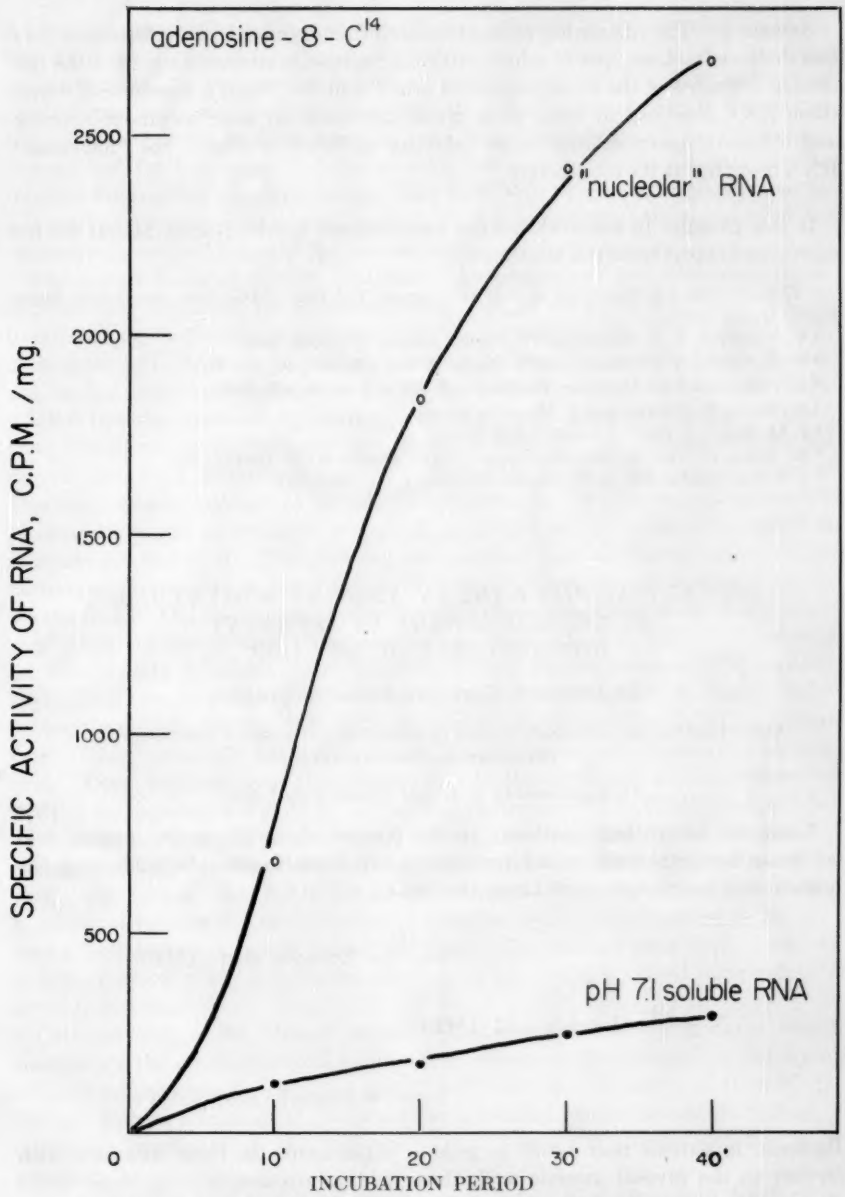

FIG. 2.—The time course of adenosine-8-C^{14} incorporation into different RNA fractions of isolated thymus nuclei. The specific activity of the RNA is plotted against the time of incubation. The incubation medium (see Allfrey, Mirsky, and Osawa, *J. Gen. Physiol.*, **40**, 451, 1957) contained 0.1 mg. (0.16 microcurie) of adenosine per 40 mg. of nuclei.

Summary.—The ribonucleic acids of isolated thymus nuclei can be separated into two distinct fractions, one of which probably represents ribonucleic acid of the nucleolus. Studies of the incorporation of orotic acid–6-C^{14} and adenosine-8-C^{14} into these RNA fractions in vitro show great differences in their metabolic activity and different susceptibilities to an inhibitor of RNA synthesis, the "nucleolar" RNA being by far the more active.

It is a pleasure to acknowledge our indebtedness to Mr. Rudolf Meudt for his careful and expert technical assistance.

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*SEQUENTIAL BLOCKADE IN ADENINE BIOSYNTHESIS BY GENETIC LOSS OF AN APPARENT BIFUNCTIONAL DEACYLASE**

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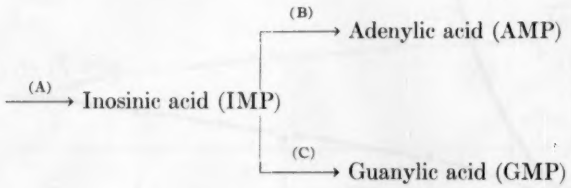
Along the biosynthetic pathway to the purines of nucleic acids, inosinic acid occurs as a pivotal point in a bifurcation which leads to adenylic acid along one branch and to guanylic acid along the other:



Bacterial mutations may result in genetic impairments at three locations with respect to the pivotal inosinic acid, thus yielding auxotrophs with three broad classes of nutritional response. A mutant which has lost the ability to synthesize inosinic acid *de novo* (block at A) can satisfy its growth requirements with various forms of hypoxanthine or, through interconversions, guanine or adenine. Such a mutant will be nondiscriminating in its choice of purines for growth and may accumulate various types of incomplete purines such as aminoimidazoles. When

a block occurs after inosinic acid, specific requirements for adenine (block at B) or for guanine (block at C) will be manifested.

The mutants to be considered here all belong to group B. Only adenine or its ribose derivatives will support their growth. The details of path B, the amination of inosinic acid to form adenylic acid, have been provided by Carter and Cohen[1] and by Lieberman.[2] Inosinic acid first combines with aspartic acid to form an intermediate, adenylosuccinic acid (AMP-S), which is then deacylated by a second enzyme to yield adenylic acid. Thus a specific growth requirement for adenine may occur if either of the two enzymes is lost by mutation.

The culture fluids of thirteen mutants of *Escherichia coli* and *Salmonella typhimurium* were examined for accumulations. Two of these accumulate a form of hypoxanthine, indicating a deficiency in the reaction which adds aspartic acid to inosinic acid. Ten of the thirteen accumulate a compound with a high absorption in the ultraviolet (maximum at 267 mμ), which at first was thought to be AMP-S but was subsequently identified as an aminoimidazole compound different from any of the aminoimidazole intermediates known to be accumulated by the nondiscriminating mutants of group A. Here, then, is an anomalous situation of a mutant which, because of its specific requirement for adenine, is apparently blocked at B, yet accumulates a type of compound which would be expected of mutants blocked at A. The paradox was clarified with the identification of the accumulated material as the ribotide of 5-amino-4-imidazole-N-succinylocarboxamide (S-AICAR), an aminoimidazole which has been implicated as an intermediate in the biosynthesis of inosinic acid.[3] S-AICAR, like AMP-S, represents an intermediate in an amination reaction formed by combination with aspartic acid. It serves as a means of introducing the amide nitrogen to 5-amino-4-imidazolecarboxamide ribotide (AICAR), eventually to become the N_1 of the purine ring. Deacylation of S-AICAR yields AICAR, the immediate precursor of inosinic acid. These reactions and their similarities to the formation and conversion of AMP-S are depicted in Figure 1. If the deacylation of both S-AICAR and AMP-S were under the control of a common enzyme, then the loss of this enzyme by mutation would create a block in the *de novo* synthesis of inosinic acid (path A) with the accumulation of S-AICAR and an additional block along branch B causing a specific deficiency in the formation of adenylic acid. The evidence to be presented indicates that this is indeed the case. The findings have been described in a preliminary note,[4] and similar conclusions have been obtained independently in other systems.[5]

Characteristics of the Mutant Strains.—The adenine-requiring mutants which accumulate the aminoimidazole include four strains of *S. typhimurium* (*ad-3* and *ad-12* obtained from T. Yura; strains 533-157 and 533-693 obtained from H. H. Plough) and six strains of *E. coli* which were isolated in this laboratory following ultraviolet irradiation of the wild-type strain B and auxotrophic selection with penicillin.

Strain B97 was selected as a typical representative of the group for further analyses. Maximum growth response is obtained with adenine or adenosine-5′-phosphate, poor growth with adenosine and adenosine-3′-phosphate, and no growth with adenosine-2′-phosphate. If 0.1 per cent casein hydrolyzate is added to the medium, then maximal growth is obtained with all the above forms except

adenosine. Hypoxanthine, guanine, isoguanine, xanthine, and their ribose derivatives are completely inert.

Maximal accumulation of the aminoimidazole can be detected in the culture fluids only under conditions of suboptimal growth, as obtained with a suboptimal supply of adenine. With an excess supply of adenine, accumulation is prevented. Rapid accumulation of the aminoimidazole can be obtained with nonproliferating cell suspensions under conditions which have been previously described.[6] With glucose and ammonium chloride as the only sources of carbon and nitrogen, as

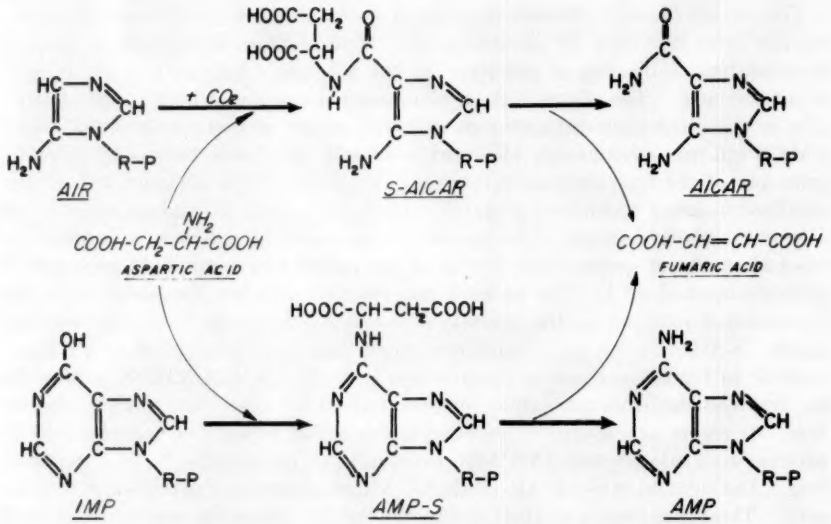


FIG. 1.—Similarities in formation and deacylation of AMP-S and S-AICAR.

much as 0.5–1.0 μmole of the amine per milliliter may be formed in 2–3 hours. Aeration is required for optimal formation, and aspartic acid, glycine, and threonine stimulate production.

Detection of S-AICAR.[7]—The accumulation of S-AICAR was first detected as a substance with a maximal absorption at 267 mμ. Subsequent assay was made by a modification of the Bratton-Marshall[8] test for diazotizable amines. This modification is based on a precise timing in the addition of the reagents and also serves to indicate that the accumulated amine is different from any of the known aminoimidazoles accumulated by other purine-requiring mutants and indeed is identical with S-AICAR. The test ordinarily calls for an interval of 5 minutes between the acidic diazotization with sodium nitrite, the removal of excess nitrite with ammonium sulfamate, and the eventual coupling with N-1-naphthylenediamine dihydrochloride to produce a stable pigment. With most arylamines, such as AICA, the timing is not critical, since the diazonium complex is stable and can produce color with the coupling reagent even after a considerable delay. S-AICAR, however, apparently forms an unstable diazonium complex which rapidly decays at room temperature, so that color can be demonstrated only with rapid

coupling. No color is obtained with the usual delay of 5 minutes. The kinetics of coupling efficiency is that of a typical first-order decay reaction with a half-life of 36 seconds. The first indication that the accumulated amine and S-AICAR were indeed identical was based on their identical rates of loss of coupling efficiency. This is depicted in Figure 2.

For assay purposes, the Bratton-Marshall test was standardized as follows: A sample of 2 ml., or an aliquot diluted to 2 ml., is acidified with 2 ml. of trichloroacetic acid (7.5 per cent); 0.4 ml. of sodium nitrite (0.1 per cent) is then added and immediately mixed. At exactly 30 seconds, 0.4 ml. of ammonium sulfamate

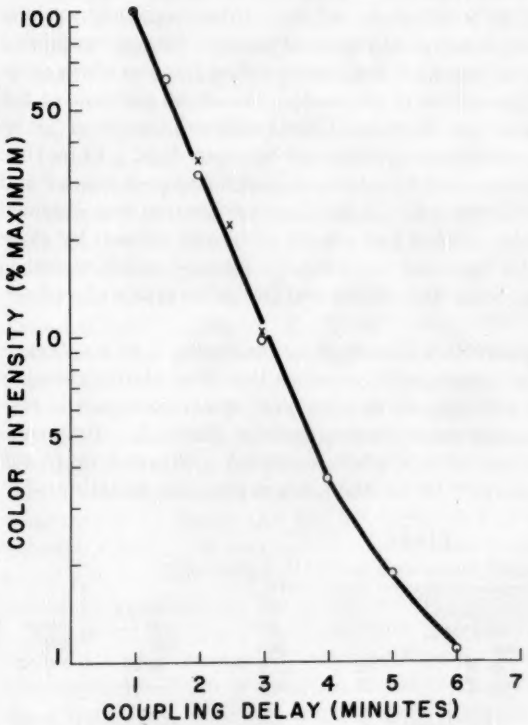


FIG. 2.—Loss of Bratton-Marshall coupling efficiency of S-AICAR and of B97 amine. Coupling delay refers to the time between diazotization and addition of coupling reagent (see text) for S-AICAR (x) and B97 amine (o).

(0.5 per cent) is added and again immediately mixed. After another 30 seconds, 0.4 ml. of N-1-naphthylenediamine dihydrochloride (0.1 per cent) is added and mixed. After 10 minutes, color intensity is read in a Klett-Summerson photometer equipped with a 540 (green) filter. A concentration of 0.1 μmole per test system gives a reading of 84 Klett units. Both the accumulated amine and S-AICAR produce a purple color with absorption maximum at 560 mμ and 540/600 optical-density ratio of 1.18.

With the Pauly test for imidazoles, as described by Koessler and Hanke,[9] the accumulated amine and S-AICAR give a fading purplish-blue color which is characteristic of aminoimidazoles. The ribose content, determined by the orcinol method

as described by Drury,[10] served as another method for assay. With a variety of manipulations and fractionations, it was impossible to separate the amine from the pentose which parallels its accumulation in equimolar amounts.

Isolation and Identification.—Washed suspensions of strain B97 were prepared by harvesting a culture grown overnight in 1.5 liters of a tryptone–yeast extract–glucose broth. The washed cells were inoculated to a concentration of 0.5 mg. of cells (dry weight) per milliliter in 1 liter of a salts-glucose medium[6] containing glucose (0.4 per cent) and ammonium chloride (0.1 per cent). Incubation was carried out with aeration, and when maximum production was obtained (3 hours), the bacteria were removed by centrifugation, and the supernatant fluid was passed over a small charcoal (Darco 20 × 40 mesh) column. After copiously washing the column with water, the amine was eluted with 50 per cent ethanol containing 20 per cent NH_4OH. The amine appeared in a visibly yellow fraction which came through soon after the first appearance of alkalinity. About 50 per cent of the total amine was recovered from the charcoal. The combined eluates of about 50 ml. which contained the amine were concentrated to about 3 ml. From this, most of the amine could be precipitated by adding several volumes of ethanol and allowing to remain overnight in the cold. Additional purification was obtained by dissolving the amine in warm alcohol and precipitating with ether. By these procedures, several batches of a light tan powder were obtained which ranged in purity (based on ribose content) from 50 to 80 per cent and gave organic phosphate-to-ribose ratios of 0.75 to 1.3.

The identity of the amine was further confirmed by comparing it with authentic S-AICAR. In addition to the colorimetric reactions described above, complete agreement was also obtained with respect to ultraviolet spectrophotometric constants (see Table 1) and ascending paper chromatography (Table 2). Both compounds are rapidly converted to AICAR when incubated with yeast or *E. coli* extracts. Acid hydrolysis with 1.0 *N* HCl for 30 minutes produces aspartic acid.

TABLE 1

COMPARISON OF B97 AMINE WITH S-AICAR (ULTRAVIOLET SPECTROPHOTOMETRIC CONSTANTS)

	B97 Amine			S-AICAR		
	pH 2	pH 7	pH 10	pH 2	pH 7	pH 10
λ max.	269.5	268	267	269.5	268	268
O.D. 250/260	0.90	0.80	0.82	0.91	0.79	0.83
O.D. 260/280	1.05	1.25	1.22	1.06	1.31	1.18

TABLE 2

COMPARISON OF B97 AMINE WITH S-AICAR (PAPER CHROMATOGRAPHY)

Solvent		R_f Values: S-AICAR	R_f Values: B97 Amine
5 per cent K_2HPO_4-isoamyl alcohol	(50:50)	0.90	0.91 (0.80)*
Ethanol-0.1 *M* acetic acid	(50:50)	0.61	0.63
n-Butanol-acetic acid-H_2O	(50:25:25)	0.25	0.25 (0.43)
Isopropanol-H_2O-NH_4OH	(70:20:10)	0.01	0.02 (0.20)

* R_f values in parentheses are for a second substance present in some preparations. This substance has been identified as the riboside of S-AICAR (see text).

As shown in Table 2, chromatography with three out of the four solvents revealed another compound whose R_f values differed from those of S-AICAR. This compound is undoubtedly the corresponding riboside of S-AICAR. It was found

primarily in those preparations with phosphate-to-ribose ratios which were significantly less than 1.0. Examination of eluates from the paper showed that it resembled S-AICAR with respect to ultraviolet spectra and pigment formation with the Bratton-Marshall reagents. The same unstable diazonium complex was evident. It differed from S-AICAR in that it was not altered by incubation with the yeast or bacterial extracts, and it contained no organic phosphate, though ribose and amine were present in equimolar amounts. Further isolations and analysis of the riboside were accomplished with strain B94, which was found to accumulate the riboside in concentrations 2–3 times greater than the ribotide.

Enzymatic Activities.—Cell-free extracts were prepared from a culture grown in 1 liter of salts-glucose medium containing adenine (30 μg/ml). After 18–24 hours of incubation with vigorous aeration, the bacteria were harvested by centrifugation, washed twice with saline, resuspended in phosphate buffer to 18 ml., and disrupted by sonic vibration in a Raytheon 9-kc. oscillator. The preparation was cleared of debris by centrifuging for 1 hour at 10,000 $\times$ g. The opalescent supernates so obtained were used for determining enzymatic activities and contained 7–10 mg. of protein per milliliter.

The conditions for determining adenylosuccinase activity were essentially the same as those described by Carter and Cohen.[1] After incubation of the extracts with adenylosuccinic acid (AMP-S),[11] the reaction products were determined by paper chromotography. The results of a typical experiment are presented diagrammatically in Figure 3. Ultraviolet-absorption analyses carried out with eluates of the spots confirmed their identities. Adenylosuccinase activity is readily demonstrated in the extract of the wild-type strain B, as evidenced by the pronounced decrease in AMP-S and the appearance of an ultraviolet-absorbing spot at R_f 0.56 which corresponds with adenosine-5′-phosphate (AMP). In addition to AMP, a faint, but distinct, ultraviolet-absorbing spot was also detectable at R_f 0.44. On the basis of its location and the spectrophotometric constants of its eluate, this has been identified as adenine, a logical degradation product of AMP. In contradistinction to these results, extracts of the auxotrophic strain B97 contain no demonstrable adenylosuccinase activity. This enzymatic deficiency would explain the specific growth requirements exhibited by strain B97.

Enzymatic splitting of S-AICAR was determined by measuring the formation of AICAR after incubation with the extracts. This could be measured by the Bratton-Marshall test by delaying coupling to 8 minutes after diazotization. Under these conditions, color formation is only obtained with AICAR. Results of a typical experiment are presented in Table 3. About 50 per cent conversion is

TABLE 3*

ENZYMATIC CONVERSION OF S-AICAR TO AICAR

Extract		S-AICAR (Initial Amt., μmoles)	AICAR (Amt. Formed, μmoles)	Per Cent Conversion
Strain	Mg. Protein			
B	1.4	0.027	0.013	48
B96	1.4	0.186	0.082	44
B97	0.8	0.027	0.000	0
B97	2.5	0.120	0.000	0

* The reaction mixture contained S-AICAR and bacterial extract in phosphate buffer (0.025 M at pH 7.2) in a total volume of 1.0 ml. Incubation was carried out at 37° for 40 minutes and stopped by addition of 1.0 ml. of 7.5 per cent trichloroacetic acid. A duplicate system which was stopped immediately after mixing the ingredients served as the zero time control.

obtained with the wild-type extract, as well as with an extract from strain B96, a nonexacting purine-requiring mutant whose genetic impairment is immediately after the formation of AICAR.[6] The extract from strain B97 is completely inactive. Under the conditions of the experiment a conversion of at least 2 per cent could have been detected.

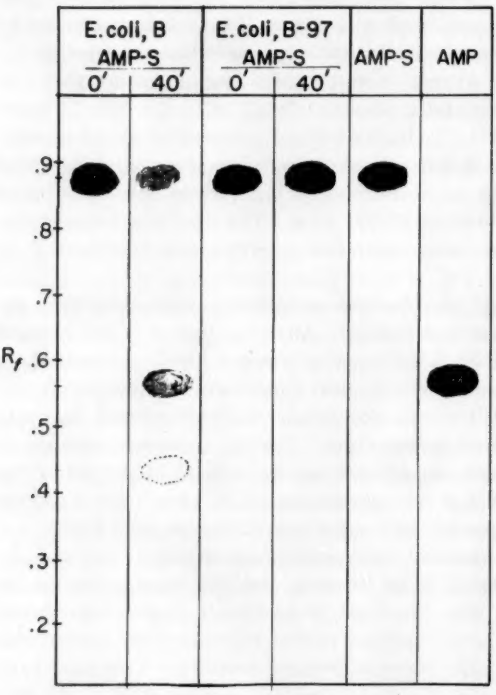


FIG. 3.—Adenylosuccinase activity. Reaction mixture consisted of extract (B, 1.4 mg. of protein; B97, 1.6 mg. of protein) and AMP-S (100 μg.) in phosphate buffer (0.025M at pH 7.2) in total volume of 1.2 ml. After 40 minutes' incubation at 37°, reaction was stopped by placing tubes in a boiling water bath for 3 minutes. Duplicate systems heated immediately after mixing the ingredients served as the zero time controls. After removal of precipitated protein by centrifugation, 0.5 ml. was run on an ascending paper chromatogram with 5 per cent K_2HPO_4–isoamyl alcohol (50:50). Spots were visualized with an ultraviolet lamp.

Discussion.—The results of these experiments offer strong indication that strain B97 differs from its wild-type progenitor by a deficiency in a common deacylase activity for both AMP-S and S-AICAR.

On the basis of the one-gene, one-enzyme hypothesis, it is assumed that this is due to the loss of a single enzyme. Thus the loss of this enzyme by a single mutational event results in the loss of two sequential reactions in the biosynthesis of adenylic acid. The double block so created prevents both the formation of inosinic acid and its conversion to adenylic acid via adenylosuccinic acid. The phenotypic consequence is an auxotrophic mutant with a specific growth requirement for adenine, which accumulates an aminoimidazole precursor, viz., S-AICAR. The existence of a multifunctional enzyme is not in itself unusual, but it is unique that the reactions controlled by the same enzyme may be found at different levels in the same biosynthetic chain.

Conclusions similar to those presented here have been drawn from independent

studies with adenine-requiring mutants of *Neurospora*.[5] With these mutants, however, the accumulation of S-AICAR was not demonstrated; instead, various derivatives of AMP-S were found in the culture fluids.[12] This finding is disturbing, since the block at the S-AICAR level should not have allowed the formation of AMP-S. It is possible, however, that the adenine supplied for the growth of the *Neurospora* mutant may have been in sufficient excess to exert a feedback inhibition of S-AICAR formation and, at the same time, to serve as a source of AMP-S via direct deamination to hypoxanthine. This complication would not enter into our studies, since they were performed with resting-cell suspensions in the absence of adenine. With the addition of hypoxanthine to these systems, we did find that S-AICAR formation was partially depressed, and the accumulation of the aglycone form of AMP-S, 6-aspartyl purine, was indeed detected.

With a block before inosinic acid, the *de novo* synthesis of guanylic acid would not be expected. Since exogenous guanine is not required for growth, the nucleic acid guanine is apparently derived from the supplied adenine. Isotopic evidence for the conversion of adenine to guanine with lack of *de novo* synthesis of both purines has been obtained with the *Salmonella* mutant, strain *ad-12*.[13] Since adenylosuccinase activity is also absent, a mechanism must exist by which adenine can be converted to guanine without involving, through reverse action, the normal biosynthetic reactions. This conversion of adenine to guanine may or may not require hypoxanthine as an intermediate, but, if it does, the hypoxanthine could well be supplied via a direct deamination of adenine. *Escherichia coli* strain B is known to contain an active adenine deaminase.[14]

Summary.—Some adenine-requiring mutants of *E. coli* and *S. typhimurium* accumulate an early biosynthetic precursor which has been identified as the ribotide of 5-amino-4-imidazole-N-succinylocarboxamide (S-AICAR). The riboside was also found. The accumulation is due to a mutational loss of a deacylating enzyme which splits S-AICAR to yield the ribotide of 5-amino-4-imidazolecarboxamide (AICAR). Loss of this activity is accompanied by a loss of the ability to deacylate adenylosuccinic acid (AMP-S) to yield adenylic acid (AMP) and hence the occurrence of a specific growth requirement for adenine. Thus the mutational loss of this apparent bifunctional deacylase creates a double block along a common biosynthetic pathway to nucleic acid adenine.

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BLUE RIDGE TECTONICS BETWEEN HARRISBURG, PENNSYLVANIA AND ASHEVILLE, NORTH CAROLINA

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The boundary between crystalline rocks to the southeast, and largely nonmetamorphic rocks to the northwest, of the Blue Ridge has been variously interpreted as a major dislocation zone, a series of abrupt flexures,[1] or a normal sequence in asymmetrical folds.[2–5] The northern half of the zone shows no important thrusts, few reverse faults, but mostly folded normal sequences. As one proceeds southward, especially south of the James River, reverse faults are numerous and thrusting becomes prevalent. The boundary between "basement" and sedimentary cover is not only an important surface of demarcation between rocks of different types and ages, probably a major break in the geologic history, but also an outstanding structural zone with distinct structures which are limited to it.

Figure 1 is a map of the area between Harrisburg, Pennsylvania, and Asheville, North Carolina, showing, much generalized, the boundary between the crystallines to the southeast and the Paleozoic folds to the northwest.[6] A distinct lineation[7] occurs along this boundary, predominantly in the older rocks, but in northern Virginia, Maryland, and Pennsylvania, the Paleozoics up to the Beekmantown[2, 8] are also lineated. The lineation is contained in a regional cleavage. The measurements shown in Figure 1 reflect thousands of field observations, especially in the northern half of the map area. The southern half has been covered by reconnaissance only, but additional field work is in progress.

Figure 1 (*inset*) also shows the Moine Thrust zone in Scotland at the same scale for comparison. Some lineation measurements have been added from various sources[9–14] to show the similarity of the pattern. The Blue Ridge zone is at least three times as long as the Moine Thrust zone. To the northeast it does not continue beyond the Susquehanna River, but it may extend beyond Asheville to the southwest. The Moine Thrust has received a great deal of attention in the literature, but the Blue Ridge comparatively very little. It is the purpose of this paper to call attention to this extraordinary structure, especially since it is well suited to test, in this particular area, some of the current concepts on lineation and deformation.

Lineation.—Lineations occur in two prominent directions: normal to and parallel with the structural trend of the Blue Ridge zone. Figure 1 emphasizes the regularity of the southeast trend, but the two directions occur side by side at many localities. Lineations which parallel the structural trend are fold axes (mostly in Paleozoic formations), intersections of cleavage and bedding, crenulations, and now

and then parallel arrangements of elongate pebbles or minerals. Normal to the trend and essentially down the dip of the planes of a regional cleavage are elongated and flattened blebs in volcanics,[4, 15, 16] elongate pebbles, streaks in schists and gneisses, the longest axes of elongated oöids in oölites,[2] and parallel orientation of minerals or spindle-shaped mineral aggregates.

Both lineations occur in basement rocks and in Paleozoics up to the Ordovician

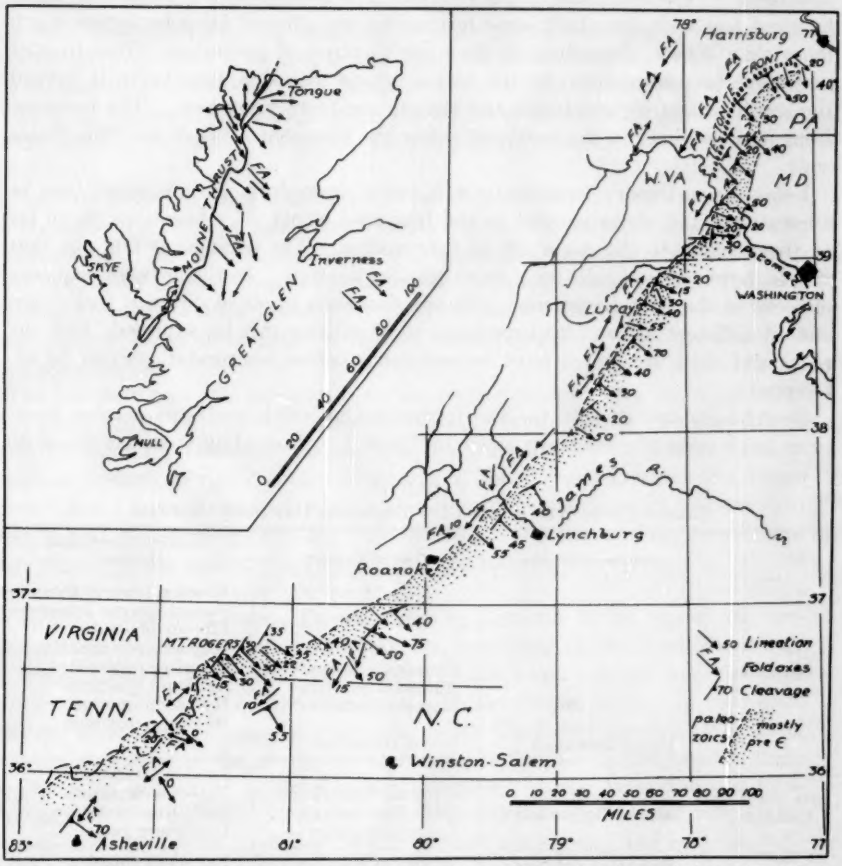

FIG. 1.—Map of Blue Ridge zone between Harrisburg, Pennsylvania, and Asheville, North Carolina. Inset: Moine Thrust zone in Scotland for comparison.

in the South Mountain area of Maryland and Pennsylvania,[8, 16, 17] in the southward continuation across the Potomac River,[4] east of Luray, Virginia,[15] and from here to the James River.[18] From Roanoke to Asheville no southeast-dipping lineations have so far been found in the lower Paleozoic rocks, except in some of the quartzites immediately overlying the basement. Proceeding upward in the section, faulting and thrusting seem to bring basement rocks in abrupt contact with unaltered lower Paleozoic sandstones and shales. As has already been shown

by King,[1] the tectonic style changes gradually and profoundly as one proceeds from the Potomac River to Asheville, North Carolina.

The petrographic boundary between basement rocks and Paleozoics is also a tectonic break in the south, whereas in the north this break lies in the Great Valley and within the Cambro-Ordovician section. Fellows[17] has called this break the "tectonite frontier," inasmuch as the rocks to its southeast are essentially tectonites and those to the northwest nontectonites. The boundary is a gradation, many hundred feet wide, in which some formations are affected by deformation much more than others, depending on their initial physical properties. This frontier may have been overridden by the basement and may disappear below it toward the south, where reverse faults and thrusts are more prominent. The tectonite front may continue to the southeast below the basement rocks of the Blue Ridge zone.

Using the customary co-ordinates *a*, *b*, and *c*, down-dip lineations would then be *a*-lineations, and those parallel to the fold axes would be *b*-lineations, in so far as they belong to the same act of deformation. The arrangement is not that simple, however, and calls for a more detailed analysis. In the complex sequence of rocks of the Blue Ridge zone, different lineations occur in different rock types and at different times. Superposition of lineations can be expected, and the rocks and their structures must be separated before interpretations can be attempted.

Stratigraphy and Lineation.—The formations in which southeast-dipping lineations have been observed are listed in Table 1. Proceeding from northeast to

TABLE 1*

FORMATIONS IN WHICH DOWN-DIP LINEATION HAS BEEN OBSERVED

	Southwest Virginia	Central Virginia	Northern Virginia and Maryland
Early Cambrian			Beekmantown limestone Conococheague limestone Elbrook dolomite Waynesboro formation
		Tomstown dolomite? Antietam quartzite? Harpers formation?	Tomstown dolomite Antietam quartzite? Harpers formation Weverton quartzite
	Unicoi formation	Unicoi formation	
Late pre-Cambrian	Mount Rogers series	Catoctin formation Swift Run formation Lynchburg gneiss	Loudon formation Catoctin formation Swift Run formation Lynchburg gneiss
	Basement complex	Basement complex	Basement complex

* Modified after Bloomer and Werner, *Bull. Geol. Soc. Amer.*, **66**, 579–606, 1955.

southwest, measurements become crowded in the crystalline rocks and are absent in the Cambrian formations, except the lowest portion of the Unicoi, for one of two possible reasons: either lack of detailed observations or the absence of such lineations in the southern Blue Ridge zone. The lithologic facies also change, and the massive sandstones and quartzites of the Smokies do not show lineations as readily as do the Catoctin greenstones.

I have searched the lower Paleozoics of southern Virginia and Tennessee for

lineations but have found that the shales and sandstones adjacent to the Blue Ridge crystallines are even without a regional cleavage. They are unaltered, and only the shales possess at best a crude and fanning fracture cleavage. The transition between schistose and well-lineated Catoctin greenstone and the Paleozoics is very abrupt.

North of the Potomac River this contact is a wide gradation in which cleavage and lineation become less intense as one proceeds upward.[2, 8, 19] A similar gradation may have existed in the south and is now covered, or it has never existed, because faulting which is lacking in the north has intervened.

Fold axes (*b*) of all orders of magnitude and intersections of cleavage and bedding occur in all Paleozoic formations to the northwest of the Blue Ridge zone and in several units within the crystallines. They are equally common in the south and in the north.

Lineation in the Catoctin Formation.—By far the most distinct down-dip lineation (*a*) occurs in the Catoctin formation. Lenticular chlorite patches are from $^1/_2$ to 4 inches long, from $^1/_{10}$ to 1 inch wide, and paper-thin, resembling willow leaves. They are rigidly aligned in cleavage planes which dip to the southeast. Between schistose greenstones occur massive layers which are not lineated, and some fairly large outcrops show no lineation at all but undeformed amygdules and fractures filled with epidote.

The orientation of the lineation is to the southeast, with very little deviation from about 120 to 130 degrees for the horizontal projection and with a dip of from 60 to 20 degrees. This lineation has been reported from South Mountain anticlinorium,[16] south of the Potomac River,[4] near Luray,[15] and north of the James River,[18] and I have observed it also in the Mount Rogers series in southwestern Virginia and in Tennessee (Fig. 1). It is consistently intense and rigidly oriented from the northeast end of the Blue Ridge structure in Pennsylvania to Mount Rogers at the Virginia-Tennessee border.

Lineation above the Catoctin Formation.—The Catoctin is the top of the pre-Cambrian in southern and central Virginia and is overlain by the Loudon in northern Virginia and in Maryland. In the south I have found only few localities with a thin veneer of lineated quartzite above the Mount Rogers series. In the north, cleavage and lineation equally affect the lower Cambrian Loudon, Weverton, Harpers, and some of the limestones above the Catoctin. In the Loudon there are elongated pebbles of all sizes in conglomerates; in the Weverton one finds ellipsoidal quartz grains and streaks; the Harpers shows ellipsoidal rust spots, streaks, and small blebs in shales; the limestones are streaky and contain oöids whose longest axes are parallel with the lineation in the Catoctin formation. The gradational decrease of deformation intensity is well shown in the axial ratios of the Cambro-Ordovician oölites.[2]

The relation of lineations to movement directions is a much-discussed problem,[20] which is by no means solved. Symmetry in the South Mountain anticlinorium indicates that the lineations in the Catoctin and above parallel the *a*-axis of the co-ordinate system. Fold axes, intersections of regional cleavage and bedding, crenulations, and the axes of quartz and mica girdle diagrams parallel the *b*-axis of the same deformation plan. The symmetry is monoclinic, and folding is overturned to the west and northwest.

Age of a-Lineation.—Only one uniform deformation plan has been found north of the Potomac River in the South Mountain anticlinorium of Maryland and Pennsylvania,[2, 5] and it has affected all formations: the Catoctin greenstones as well as the Cambro-Ordovician limestones of the Great Valley. The deformation which produced the *a*-lineation in that area must therefore be post-Ordovician, that is, either Taconic or Appalachian. In view of the astounding regularity of orientation along the entire Blue Ridge zone and the similarity of the lineation of the Catoctin formation from Pennsylvania to the James River to that of the Mount Rogers series along the Virginia-Tennessee border, I think that the *a*-lineation along the entire Blue Ridge zone is post-Ordovician, even if that cannot be proved in the south. It is possible that a systematic study of oölite deformation south of the Potomac River and along the Great Valley would contribute materially to the solution of this problem.

a-Lineation below Catoctin Formation.—Below the Catoctin formation are the Swift Run of northern and central Virginia, and a series of gneisses, granites, and schists. A distinct *a*-lineation occurs in some of these, as, for instance, in the Swift Run formation.[18] In the gneiss along the Potomac River a very intense lineation occurs parallel to that in the Catoctin above. It is, however, strong only in limited shear zones within massive gneiss. Proceeding southward, especially south of the James River, fold axes, crenulations, intersections, and mineral parallelism occur in the gneisses along with lenticular mineral aggregates, patches, and striations, suggesting not only *a*- but also *b*-axes in the same direction or at various angles to it. South of the Virginia-Tennessee border, orientations are much less regular, and the pattern is very suggestive of the superposition of a later cleavage and lineations on older patterns. A great deal of more detailed work needs to be done in this portion of the Blue Ridge zone.

Blue Ridge and Moine Thrust.—In the Moine Thrust area at least two major directions compete with each other: one parallel with the general trend and another one across it. Both show lineations, especially fold axes, crenulations, rodding, intersections, and axes of girdles in fabric diagrams. Patterns of different ages have been superimposed, and axes have been refolded. Similar rocks occur on both sides of the thrust zone, and similar intensities of metamorphism. The simple pattern of the northern Blue Ridge zone (South Mountain fold) does not occur in Scotland, but the Moine Thrust zone resembles the southern Blue Ridge southeast of the Paleozoics. Extensive thrusting occurs along the entire Moine Thrust zone but is limited to the southern half of the Blue Ridge zone.

Summary and Conclusions.—The Blue Ridge zone is a tectonic seam of the first order, comparable to and much longer than the Moine Thrust. It stands out not only as a major geologic boundary between rocks of different composition and ages but is emphasized by a lineation and regional cleavage which can be recognized along its entire trend. In the southern half of the zone, thrusts and intensely telescoped folds dominate the tectonic style. Proceeding northward, major thrusts become fewer and finally disappear, and the zone terminates in a wide asymmetrical anticlinorium with normal sequences and gradations. Toward the Susquehanna River the structure disappears below Cambro-Ordovician limestones.

The Blue Ridge zone is uniquely suited for a systematic study of the origin of

lineations in relation to deformation, cleavage, and thrusting. The tectonite frontier continues to the south and may disappear below the crystallines, because it has been overridden in the southern Appalachians. Lineation studies south of the James River may well illuminate the problem of *a*- and *b*-lineations and superposition of different ages. To my knowledge, no other similar major structure has been described in America, and it would seem that such a large and varied tectonic unit deserves a great deal more attention than it has received in the past.

* The author is gratefully indebted to the John Simon Guggenheim Foundation for generous financial support of his work on Appalachian structures.

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FUNCTIONAL EQUATIONS IN THE THEORY OF DYNAMIC PROGRAMMING

VIII. THE VARIATION OF GREEN'S FUNCTIONS FOR THE ONE-DIMENSIONAL CASE

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1. *Introduction.*—Consider the inhomogeneous linear differential equation

$$\begin{aligned} &u'' + q(x)u = v(x), \qquad a < x < 1, \\ &u(a) = u(1) = 0 \end{aligned} \tag{1.1}$$

whose solution, under the assumption that 1 is not a characteristic value of the corresponding Sturm-Liouville problem, can be represented in the form

$$u(x) = \int_a^1 K(x, y)\, v(y)\, dy. \tag{1.2}$$

The kernel of the linear functional is called the Green's function of the operator $u'' + q(x)u$.

Since $K(x, y)$ depends upon the interval, which is to say, in this case, upon the quantity a, it is of interest to study the analytic dependence of $K(x, y)$ upon a. This study was initiated by Hadamard and continued by Schiffer. An excellent expository account of the type of results that may be obtained is given by Schiffer.[1]

In this note we wish to indicate a new approach to questions of this type, using the functional-equation technique of dynamic programming.[2]

We shall use the second-order ordinary differential equation to illustrate our method, leaving the consideration of the corresponding results for partial differential equations for further papers which will require a certain amount of the theory of functionals.

2. *Preliminaries.*—Proceeding formally, we observe that

$$\max_u [(u, Au) - 2(v, u)] = (A^{-1}v, v), \tag{2.1}$$

with $u = A^{-1}v$ yielding the maximum. Identifying Au with $u'' + q(x)u$ and $A^{-1}v$ with $\int_a^1 K(x, y)\, v(y)\, dy$, we wish to study the variation of $K(x, y) = K(x, y, a)$ with a by studying the dependence of the maximum of

$$J(u) = \int_a^1 [u(u'' + q(x)u) - 2vu]\, dx = \int_a^1 [q(x)u^2 - u'^2 - 2vu]\, dx$$

over all u satisfying the conditions $u(a) = u(1) = 0$, upon a. In order to insure the existence of this maximum, let us assume that $q(x)$ is less than the smallest characteristic value of the Sturm-Liouville problem for the interval $[0, 1]$ and that $0 < a < 1$.

3. *Functional Equations and Partial Differential Equations.*—In order to carry out the above plan, we employ the functional-equation technique of dynamic programming.[1, 2] Define the function

$$f(a, c) = \max \int_a^1 [q(x)u^2 - u'^2 - 2vu]\, dx, \tag{3.1}$$

where the maximum is taken over the functions constrained by the conditions $u(a) = c$, $u(1) = 0$. It follows that $f(a, c)$ satisfies the nonlinear partial differential equation

$$\frac{\partial f}{\partial a} = q(a)c^2 - 2v(a)c + \frac{(\partial f/\partial c)^2}{4}. \tag{3.2}$$

To express $f(a, c)$ in terms of $(A^{-1}v, v)$, let us make the change of variable $u = c(1 - x)/(1 - a) + w$, which yields $w(a) = w(1) = 0$. Then

$$J(u) = \int_a^1 \left[w'^2 - q(x)w^2 + \frac{2w(v - cq(x)(1 - x))}{1 - a} \right] dx + \frac{2c}{1 - a} \int_a^1 v(x)(1 - x)\, dx + c^2 \left[(1 - a) - \int_a^1 \frac{q(x)(1 - x)^2}{(1 - a)^2}\, dx \right]. \tag{3.3}$$

Thus, referring to Section 2,

$$f(a, c) = c^2\left[(1 - a) - \int_a^1 \frac{q(x)(1 - x)^2}{(1 - a)^2}\,dx\right] + 2c\int_a^1 \frac{v(x)(1 - x)}{1 - a}\,dx\Bigg] -$$
$$\int_a^1\int_a^1 K(x, y, a)\left[v(y) - \frac{cq(y)(1 - y)}{1 - a}\right]\left[v(x) - \frac{cq(x)(1 - x)}{1 - a}\right]dx\,dy. \quad (3.4)$$

It follows that we can write

$$f(a, c) = \int_a^1\int_a^1 K(x, y, a)\,v(x)\,v(y)\,dx\,dy + 2cL_1(v) + c^2g(a),$$

where $g(a)$ is a function independent of c and $v(x)$ and $L_1(v)$ is a linear functional of v given by the expression

$$L_1(v) = \int_a^1 \frac{v(x)(1 - x)}{1 - a}\,dx + \int_a^1\int_a^1 K(x, y, a)\,q(y)\left(\frac{1 - y}{1 - a}\right)v(x)\,dx\,dy. \quad (3.5)$$

Combining the expression for $f(a, c)$ given by equation (3.5) with the nonlinear partial differential equation of equation (3.2), we obtain, upon equating the coefficients of c^2, the relation

$$4L_1(v)^2 = -\int_a^1\int_a^1 \frac{\partial K}{\partial a}\,v(x)\,v(y)\,dx\,dy \quad (3.6)$$

(recalling that $K(a, y, a) = K(x, a, a) = 0$). Since we may write

$$4L_1(v)^2 = 4\int_a^1\int_a^1\left[\frac{1 - x}{1 - a} + \int_a^1 \frac{K(x, z_1, a)}{1 - a}\,q(z_1)(1 - z_1)\,dz_1\right]\times$$
$$\left[\frac{1 - y}{1 - a} + \int_a^1 \frac{K(y, z_2, a)}{1 - a}\,q(z_2)(1 - z_2)\,dz_2\right]v(x)\,v(y)\,dx\,dy, \quad (3.7)$$

we obtain, upon "equating coefficients" of $v(x)\,v(y)$, the relation

$$\frac{\partial K(x, y, a)}{\partial a} = -4\left[\frac{1 - x}{1 - a} + \int_a^1 \frac{K(x, z_1, a)}{1 - a}\,q(z_1)(1 - z_1)\,dz_1\right]\times$$
$$\left[\frac{1 - y}{1 - a} + \int_a^1 \frac{K(y, z_2, a)}{1 - a}\,q(z_2)(1 - z_2)\,dz_2\right]. \quad (3.8)$$

This is the desired formula expressing the variation of $K(x, y, a)$ with a.

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TWO THEOREMS IN GRAPH THEORY

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Introduction.—Given an unoriented graph (or 1-dimensional regular complex), let X be the set of all its vertices and U be the set of all its edges. When the graph is finite, the following problems arise:

Problem 1: A set $A \subset X$ is said to be *internally stable* if $x \in A$, $y \in A$ implies $(x, y) \notin U$. The symbol $|A|$ will denote the number of elements of A. Construct an internally stable set A such that $|A|$ is maximum.

Problem 2: A set $B \subset X$ is said to be a *cover* if every edge of U is adjacent to at least one vertex in B. Construct a cover with the minimum number of elements.

Problem 3: A set of edges $V \subset U$ is said to be a *matching* if two edges of V have no vertex in common. Construct a matching with the maximum number of elements.

A particular case of Problem 1 is the chess problem of Gauss: Put eight queens on the board such that no one can take any other. In n-person game theory, if the graph of domination is symmetrical, a maximum internally stable set turns out to be a maximum *solution* (in the von Neumann-Morgenstern sense[1]), and the more usual case can be solved by means of the Grundy functions.[2]

Problem 2 is the set theoretic dual of Problem 1, since the complement of an internally stable set is a cover, and conversely. Particular cases of Problem 3 are the problem of distinct representatives (P. Hall[3]) and the problem of Petersen (D. König[4]). In the case where the graph is bipartite, Problem 3 has been solved by algebraic methods by O. Ore,[5] and an efficient algorithm has been given by H. Kuhn.[6] Unfortunately, the linear programming duality used by H. Kuhn no longer subsists when the graph is not bipartite. (Note that Problem 2 is the linear program dual to Problem 3 in the bipartite case.) In view of solving the general case, this paper states two theorems: Theorem 1 gives a necessary and sufficient condition for recognizing whether a matching is maximum and provides an algorithm for Problem 3, while Theorem 2 yields an algorithm for Problems 1 and 2.

The Theorems.—Consider a graph $G = (X, U)$ with a matching V_0; if $u \in V_0$ we shall say that edge u is *strong*, otherwise that u is *weak*. An *alternating chain* is a chain which does not use the same edge twice and is such that for any two adjacent edges one is strong and the other is weak. A vertex x which is not adjacent to a strong edge is said to be *neutral*, the set of all neutral points being N.

We shall also consider a graph $\bar{G}$ constructed from G by adding a vertex $\bar{a}$ and connecting $\bar{a}$ to every neutral point with a strong edge. If there exists an alternating chain from $\bar{a}$ to a vertex x, we shall picture an arrow on the last edge (z, x), directed from z to x. A vertex x ($\notin N$) which is not adjacent to a directed edge is said to be *inaccessible*, the set of all inaccessible points being I. A vertex x ($\notin N$) adjacent to a weak edge directed to x and not to a strong edge directed to x is said to be *weak*, the set of all weak points being W. A vertex x ($\notin N$) adjacent to a strong edge directed to x and not to a weak edge directed to x is said to be *strong*,

the set of all strong points being S. A vertex x ($\notin N$) adjacent to a strong edge directed to x and to a weak edge directed to x is said to be *medium*, and the set of all medium points will be designated by M.

LEMMA 1. *Let Y be a connected component of the subgraph M; if $\bar{a}$ is inaccessible, there exists in G one strong edge adjacent to Y and directed to Y only; all other edges adjacent to Y are weak and directed from Y only. Moreover, all vertices not in Y and connected to Y by one edge are weak, and $|Y| \geq 3$.*

This is a theorem of T. Gallai;[7] a shorter proof is given by Berge.[8]

LEMMA 2. *If $\bar{a}$ is inaccessible, $S \cup N$ is internally stable.*

(Immediate.)

LEMMA 3. *If $\bar{a}$ is inaccessible, $M = \phi$ and $I = \phi$, then $S \cup N$ is a maximum internally stable set, W is a minimum cover, and V_0 is a maximum matching.*

From Lemma 2, $S \cup N$ is internally stable, hence $W = X - (S \cup N)$ is a cover. For every cover C and for every matching V, one has $|C| \geq |V|$; as $|W| = |V_0|$, the cover W is minimum and the matching V_0 is maximum.

LEMMA 4. *Let Z be a connected component of the subgraph I; if $\bar{a}$ is inaccessible, all edges adjacent to Z are weak and undirected; moreover, all vertices not in Z connected to Z by an edge are weak, and $|Z| \geq 2$.*

(Immediate.)

LEMMA 5. *If $|N| \leq 1$, V_0 is a maximum matching.*

This follows from the fact that $|X| = 2|V_0| + |N|$.

LEMMA 6. *If $A \subset X$, let G_A be the graph constructed from G by shrinking A into a single vertex a_A, having as adjacent edges the adjacent edges of A. If the original strong edges constitute a maximum matching for the subgraph A, and for G_A, then V_0 is a maximum matching for G.*

This is easy to see by an induction on the number of elements of A.

THEOREM 1. *A matching V is maximum if and only if there does not exist an alternating chain connecting a neutral point to another neutral point.*

If there existed an alternating chain $W = (u_1, u_2 \ldots, u_k)$ connecting a neutral point a to a neutral point a' different from a, $(V - W) \cup (W - V)$ would be a matching with more elements than V, and V would not be maximum.

Conversely, let us prove that, if such a chain does not exist, V is maximum; the proposition being obvious when the graph has one or two edges, we shall assume that the proposition is true for any graph having fewer than m edges, and we shall prove it for a graph G of m edges. One can assume that G is connected.

From Lemma 5, one can assume $|N| > 1$; from Lemma 3, one can also assume that either $M \neq \phi$ or $I \neq \phi$.

1. If $M \neq \phi$, let Y be a connected component of the subgraph M; the graph G_Y constructed from G by shrinkage satisfies the conditions of the theorem (Lemma 1); as it has at least one edge less than G, the strong edges constitute a maximum matching for G_Y. On the other hand, the subgraph Y has only one neutral point (Lemma 1) and therefore its strong edges constitute a maximum matching. Thus, from Lemma 6, V_0 is a maximum matching for G.

2. If $I \neq \phi$, let Z be a connected component of subgraph I, and consider the graph G_Z. The vertex a_Z is a neutral point, connected only with weak points. No alternating chain leads from a point of N to a_Z. As G_Z satisfies the conditions of the theorem, G_Z admits its strong edges as a maximum matching. On the

other hand, the subgraph Z, having no neutral points, admits its strong edges as a maximum matching; therefore, V_0 is a maximum matching for G.

THEOREM 2. *Let C_Y (resp. C_Z) be any minimum cover for the subgraph generated by a connected component Y of M (resp. Z of I). If there does not exist an alternating chain connecting a neutral point to another neutral point, the set*

$$C = W \cup \bigcup_Y C_Y \cup \bigcup_Z C_Z$$

is a minimum cover for G.[9]

Every vertex which is connected by an edge to a component Y is a weak point (Lemma 1); every vertex which is connected by an edge to a component Z is a weak point (Lemma 4). Therefore C is a cover for G. As C is a minimum cover for the graph G' deduced from G by removing all edges connecting a weak vertex to a medium or inaccessible vertex (Lemma 3), C is also a minimum cover for G.

Theorem 1 suggests the following procedure for solving Problem 3; Construct a maximal matching V, and determine whether there exists an alternating chain W connecting two neutral points. (The procedure is known.) If such a chain exists, change V into $(V - W) \cup (W - V)$, and look again for a new alternating chain; if such a chain does not exist, V is maximum.

Theorem 2 gives an algorithm for Problem 2, hence for Problem 1.

* Princeton University and C.N.R.S., Paris. This study was prepared at the Economics Research Project, Princeton University, under contract with the Office of Naval Research.

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*MATHEMATICS: POLYNOMIALS OF BEST APPROXIMATION ON A REAL FINITE POINT SET**

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Polynomials $p_n(x)$ of given degree n (≥ 0) of best approximation to a given function on a real finite point set E: $(x_1, x_2, \ldots, x_m)$ are important in numerical computation; they have various properties in common, especially those relating to oscillation of the difference $f(x) - p_n(x)$ on E; here approximation is measured according to any of the classical deviations, such as that of Tchebycheff,

$$\max\,[\mu_k|f(x_k) - p_n(x_k)|,\ k = 1, 2, \ldots, m] \qquad \mu_k > 0,$$

or that of least pth powers,

$$\sum_{k=1}^{m} \mu_k|f(x_k) - p_n(x_k)|^p, \qquad \mu_k > 0, \qquad p > 0.$$

The object of the present note is to state without proof some new results on the totality of such polynomials; detailed proofs are closely related to previous (since 1953) joint work by the writers and will be published elsewhere.

We say that the polynomial $q_n(x) \equiv b_0x^n + b_1x^{n-1} + \ldots + b_n$ of degree n is a *closer* polynomial to $f(x)$ on E than the polynomial $p_n(x) \equiv a_0x^n + a_1x^{n-1} + \ldots + a_n$ of degree n, provided that $q_n(x) \not\equiv p_n(x)$ and provided that

$$|f(x) - q_n(x)| < |f(x) - p_n(x)| \text{ on } E \text{ where } f(x) - p_n(x) \neq 0, \tag{1}$$

$$f(x) - q_n(x) = f(x) - p_n(x) \text{ on } E \text{ where } f(x) - p_n(x) = 0. \tag{2}$$

If $p_n(x)$ admits no closer polynomial to $f(x)$ on E, it is called a *juxtapolynomial* to $f(x)$ on E.

If E contains fewer than $n + 1$ points, there exist infinitely many polynomials of degree n coinciding with $f(x)$ on E, a case henceforth excluded.

If $p_n(x)$ is a juxtapolynomial to $f(x)$ on E, it is also a juxtapolynomial to $f(x)$ on every set containing E. A polynomial of best approximation to $f(x)$ on E in any of the classical senses already mentioned is clearly a juxtapolynomial to $f(x)$ on E.

In the special case $f(x) \equiv x^{n+1}$, the polynomial $x^{n+1} - p_n(x)$ is called an *extremal* polynomial or *T-polynomial* on E if it minimizes a deviation (norm), and if equations (1) and (2) are impossible, it is called an *infrapolynomial* on E.

We say that a function $\varphi(x)$ *oscillates weakly* on the real set E: $(x_1, x_2, \ldots, x_\lambda)$, where $x_1 < x_2 < \ldots < x_\lambda$, provided that we have one of the following two sets of inequalities:

$$\varphi(x_1) \geq 0, \quad \varphi(x_2) \leq 0, \quad \varphi(x_3) \geq 0, \ldots,$$

$$\varphi(x_1) \leq 0, \quad \varphi(x_2) \geq 0, \quad \varphi(x_3) \leq 0, \ldots;$$

a pair of adjacent inequalities here represents a *weak sign change* of $\varphi(x)$, which is a limiting case of the usual (strong) sign change.

A continuous function $\varphi(x)$ has a *double zero* at any point X with $\varphi(X) = 0$ if it

does not change sign there, in the sense that $\varphi(x) \geq 0$ or $\varphi(x) \leq 0$ throughout some neighborhood of X.

LEMMA 1. *If $\varphi(x)$ is continuous for $x_1 \leq x \leq x_\lambda$ and oscillates weakly on the set E: $(x_1, x_2, \ldots, x_\lambda)$, then there exist zeros y_k of $\varphi(x)$ such that we have*

$$x_1 \leq y_1 \leq x_2 \leq y_2 \leq \ldots \leq y_{\lambda-1} \leq x_\lambda.$$

That is to say, a suitable set of $\lambda - 1$ zeros of $\varphi(x)$ separates weakly the points of E.

LEMMA 2. *A polynomial $p_n(x)$ of degree n which has at least $n + 1$ weak sign changes on a set $x_1, x_2, \ldots, x_{n+2}$ vanishes identically.*

THEOREM 1. *Let the real function $f(x)$ be defined on the real finite set E containing at least $n + 2$ points. The three classes of polynomials $p_n(x)$ of degree n defined by each of the following properties are identical:*

1. *$f(x) - p_n(x)$ has at least $n + 1$ weak sign changes on E.*
2. *$p_n(x)$ is a juxtapolynomial to $f(x)$ on E.*
3. *$p_n(x)$ is a polynomial of best approximation to $f(x)$ on E in the sense of least first powers with suitable positive weights.*

It is customary to denote weighted Tchebycheff approximation by least pth powers, $p = \infty$, for it can be interpreted as a limit of the case of finite p as $p \to \infty$. In this terminology we have also

THEOREM 2. *Let the real function $f(x)$ be defined on the real finite set E containing at least $n + 2$ points, and suppose $f(x)$ not identical on E to any polynomial of degree n. The three classes of polynomials $p_n(x)$ of degree n defined by each of the following properties are identical, and property 3 is independent of p $(1 < p \leq \infty)$:*

1. *$f(x) - p_n(x)$ has at least $n + 1$ strong sign changes on E.*
2. *$p_n(x)$ is a proper (i. e., pointwise different from $f(x)$) juxtapolynomial to $f(x)$ on some subset of E containing $n + 2$ points.*
3. *$p_n(x)$ is a polynomial of best approximation to $f(x)$ on E in the sense of least pth powers $(1 < p \leq \infty)$ with some positive weights.*

THEOREM 3. *Let E be a real finite set containing at least $n + 2$ points. The three classes (necessarily finite) of polynomials $p_n(x)$ of degree n defined by each of the following properties are identical, and property 3 is independent of p $(0 < p < 1)$:*

1. *$f(x) - p_n(x)$ vanishes in at least $n + 1$ points of E.*
2. *$p_n(x)$ is a juxtapolynomial to $f(x)$ on E equal to $f(x)$ in at least $n + 1$ points of E.*
3. *$p_n(x)$ is a polynomial of best approximation to $f(x)$ on E in the sense of least pth powers $(0 < p < 1)$ with some positive weights.*

Theorems 1, 2, and 3 have application to the study of infrapolynomials.

If the function $f(x)$ on the real finite set E containing at least $n + 2$ points is given, the corresponding set J of juxtapolynomials is connected, closed, and bounded.

Let the function $f(x)$ on the real finite set E containing at least $n + 2$ points be given; represent an arbitrary real polynomial by its coefficients as co-ordinates in $(n + 1)$-space. Then the set J (if not a single point) is polyhedral, and its interior is not empty and is connected and coincides with the classes defined in Theorem 2.

If here E consists of precisely $n + 3$ points, there exists a unique polynomial which is a juxtapolynomial to $f(x)$ on every subset of $n + 2$ points.

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*SPECIFICITY OF INTERMOLECULAR FORCES DUE TO QUANTUM-MECHANICAL AND THERMAL CHARGE FLUCTUATIONS**

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The preceding note[1] (Paper I) developed the theory of the specificity of some intermolecular forces: the forces due to fluctuations of electric charge distribution in and over the interacting molecules. It was found that for a mixture of molecules in a liquid medium these forces cause a rearrangement of the molecules, so that molecules which have the same distribution of oscillator polarizabilities and oscillator orientations over the frequency spectrum tend to associate. This means that like molecules tend to become nearest neighbors. For that reason it was said that there is specific attraction between identical molecules.

The purpose of this note is to estimate the magnitude of the forces and to consider in greater detail the biological implications of their specificity.

Estimate of the Magnitude of the Interaction.—In Paper I the discrimination between two molecule types, I and II, was schematically illustrated. For that purpose a figure was drawn[2] which refers to a hypothetical case in which one molecule has only oscillators in one frequency region, the other molecule only oscillators in another frequency region. In the general case each molecule may have its polarizable oscillators distributed all over the frequency spectrum. The question arises: Where are the most important frequency regions which contribute to these interactions? In the actual biologically interesting cases the most important polarizabilities might be in several ultraviolet regions ($\hbar\omega_i \gg kT$) and in "classical" low-frequency regions ($\hbar\omega_i \ll kT$).

The ultraviolet contributions to the polarizabilities are evidently contributions from electronic oscillators (mainly valence shell electrons whose possible transitions can be represented by oscillators). The quantum-mechanical zero-point fluctuations of these oscillators produce the London-Eisenschitz-Wang force. The classical low-frequency contributions to the polarizabilities can be attributed to fluctuations of the distribution of mobile protons over the surfaces of the molecules, as investigated by Kirkwood and Shumaker.[3] The theory of specificity developed in Paper I readily permits inclusion of the Kirkwood-Shumaker force because it falls, together with the London force, into the category of forces which occur when charge fluctuations are present.

In order to get at a crude estimate of a minimum size of the interaction energies involved, we might, as in Paper I, again study one-dimensional oscillators. We might then combine all their ultraviolet polarizabilities $\Sigma\alpha_{UV}$ and assign to them an average frequency, $\hbar\omega_{UV}/2\pi kT \approx 80$, and, on the other hand, combine all their low-frequency "classical" polarizabilities, $\Sigma\alpha_{Cl}$, corresponding to $\hbar\omega_{Cl}/2\pi kT \approx 10^{-6} \approx 0$. In an actual case, we will certainly find a distribution of polarizabilities over a wide frequency spectrum, and we will have anisotropy in the directional distribution of the oscillators, all of which contributes to the specificity of the interaction.

Consider the closest approach between neighboring molecules so that

$$R = \left(\frac{6 \text{ volume}}{\pi}\right)^{1/3} \tag{1}$$

is the separation of their centers in terms of the volumes of the interacting molecules. If a manifold of molecular types II is considered in interaction with a particular molecular type I and if, for simplicity, I is chosen to be an average type of the manifold, the average (over the manifold) rearrangement free energy is

$$\langle -\Delta_4 A_{\mathrm{I\,II}} \rangle_{\mathrm{Av}} \approx \text{mean-square deviation of } \left[2kT\left(\frac{\hbar\varpi_{\mathrm{UV}}}{4kT}\right)\right]^{1/2}\left(\frac{\pi}{6 \text{ volume}}\right)\sum\alpha_{\mathrm{UV}} \text{ plus that of } [2kT]^{1/2}\left(\frac{\pi}{6 \text{ volume}}\right)\sum\alpha_{\mathrm{Cl}} \tag{2}$$

It is only the circumstance that the frequencies ϖ_{UV} and ϖ_{Cl} are so entirely different which permits $-\Delta_4 A_{\mathrm{I\,II}}$ to be approximately equal to a sum over these two frequency regions (see Yos, Bade, and Jehle[4] for a more detailed discussion). Without going into the detailed assumptions about the *manifold* of molecular types under consideration, one can estimate the squares of the quantities listed in formula (2) for typical molecules instead of their mean-square deviations for a manifold of molecules. A look at Figure 1 and equation (9) of Paper I clarifies the difference between these two estimates.

As a first step in an attempt to estimate the size of the *ultraviolet* terms, we can add up the atomic static polarizabilities of the atoms occurring, e.g., in a glycine residue, and then get for a residue, $\Sigma\alpha_{\mathrm{UV}} \approx 7.5 \cdot 10^{-24}$ cm.^{3} (taking, as effective oscillator strength, Σf, one-half the number of electrons in the valence shells, i.e., 11). The frequency is estimated from the ionization energy as $\varpi_{\mathrm{UV}} \approx 2 \cdot 10^{16}$ sec.$^{-1}$ and the volume per residue $\approx 60 \cdot 10^{-24}$ cm.^{3}. This makes the square of the ultraviolet terms listed in formula (2) $\approx kT$.

Actually, it is necessary to study molecular electronic states and their polarizabilities rather than atomic polarizabilities. Of particular interest are electronic transitions in molecules which correspond to high electron mobility. There are circumstances which might greatly enhance the importance of ultraviolet terms and make them much stronger than kT, in particular the occurrence of low-frequency electronic transitions or the presence of excited electronic states which are in reach of thermal excitation. The quantity $\varpi(\Sigma\alpha)^2$ which characterizes the square of the ultraviolet term listed in formula (2) is proportional to $\varpi^{-3}(\Sigma f)^2$. The total Σf is, by the Thomas-Reiche-Kuhn sum rule (Kramers and Kronig[5]), limited by the number of electrons. A shift of the oscillator strengths toward lower frequencies therefore intensifies the interaction. It also provides for a distribution of polarizabilities over several ultraviolet regions and thereby enhances the specificity.

For large molecules, each of which presents a large number of repetitions of monomer units, other effects might still come into play which will, because of higher electron mobility, again substantially raise the ultraviolet term for which kT was estimated as a minimum in the case of a glycine residue.

In the *classical*, i.e., *low-frequency*, region the Kirkwood-Shumaker proton fluctua-

tions play a decisive role. Even though these are not oscillations but simply fluctuations with relaxation times of the order of 10^{-8} second, part of their influence is like that of classical polarizable oscillators. At separations R given by equation (1), the Kirkwood-Shumaker dipole-moment fluctuations are equivalent to an addition of low-frequency polarizability, equal to the mean-square deviation of the dipole moment divided by $3kT$. Using the data given in the papers by Kirkwood and Shumaker, one may get, for the square of the classical term listed in formula (2), a value of the order of kT for a molecule of the size of an amino acid residue.

The Kirkwood-Shumaker forces depend on the right kind of ionic concentrations in the medium. Even if these conditions are not quite satisfied, the influence of those forces is still expected to be important. One has to remember that static electric charge distributions on the interacting molecules (which may cause repulsion, particularly if the molecules are identical), are readily compensated by small ions from the medium; fluctuations of proton distributions are not easily compensated when the interacting molecules are near each other, even if the ionic concentration in the medium is considerable. (Electronic fluctuations are never compensated, of course; they are too fast.)

For a larger molecule of the type of a human serum albumin, the classical Kirkwood-Shumaker term (2) becomes of the order of 65 kT. The dependence of the interaction on the size of the molecule may be inferred from Kirkwood and Shumaker[3] (p. 858, eq. [7], and p. 869, eq. [13]).

Here, as in the ultraviolet, the larger molecules have a chance of having a stronger interaction energy, at closest-neighbor separations given by equation (1). This is true because the total polarizability of a larger molecule as compared with that of a small molecule is in actuality greater than the ratio of their volumes would indicate. (If the molecular polarizabilities were to be obtained additively from the atomic polarizabilities, they would be proportional to the volume as indicated in Paper I.)

Biological Implications.—The duplication process of a Watson-Crick DNA helix is illustrated in Figures 1*a*–1*d*. The medium surrounding the parent helix supplies nucleotides or polynucleotides. Brownian motion carries them rapidly around. Specific London interaction causes the retention of those nucleotides which happen to be identical with the nucleotides of the parent helix, in locations adjacent to the corresponding nucleotides of the parent helix, i.e., in a mantle region (an annular cylinder) surrounding the parent helix. The London force causes a particular orientation of the daughter nucleotides with respect to those of the parent helix, as shown in Figures 1*a*–1*d*.

These figures show a section of four nucleotide layers of a parent DNA helix (fartherest away from the camera an adenine, then another adenine, then an adenine-thymine pair, and another adenine-thymine pair). They also show one pair of nucleotides, (a thymine-adenine pair), selectively collected from the medium, which is the first piece of a daughter DNA molecule in process of formation. It is located in the "mantle region" surrounding the parent helix. This daughter nucleotide pair occupies the energetically favorable orientation with respect to the corresponding adjacent nucleotide pair of the parent helix; one figure shows that daughter nucleotide pair approaching, the other figure shows it adjacent to the parent nucleotide pair. Only one nucleotide layer of the daughter helix is shown;

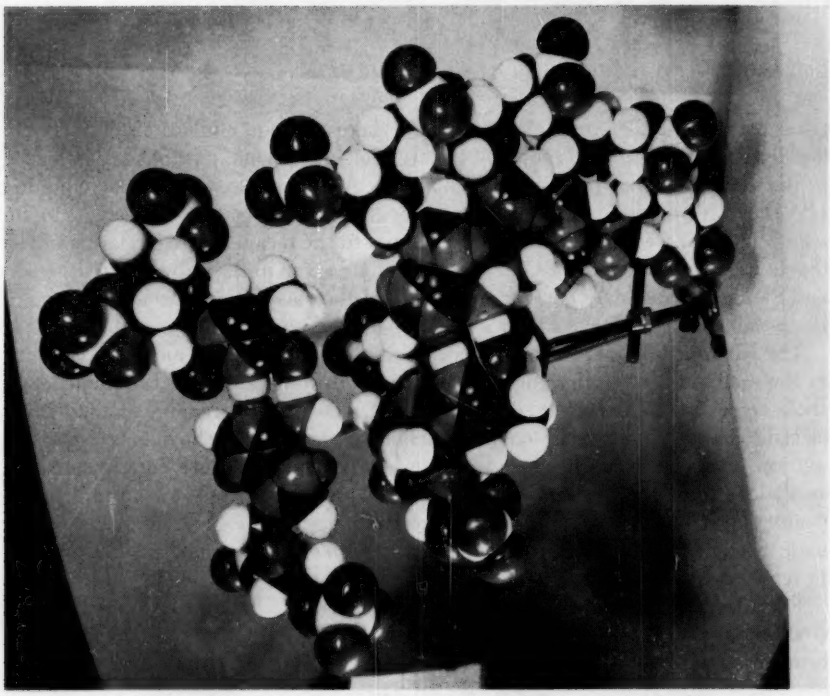

FIG. 1*a*

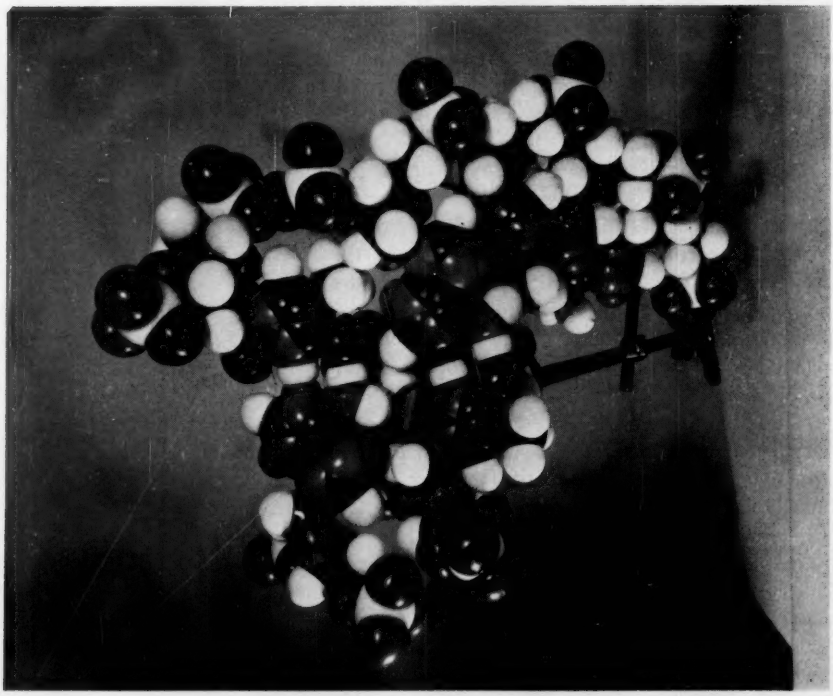

FIG. 1*b*

FIGS. 1*a*–1*d*.—One possible scheme for DNA replica formation. Figs. 1*a*, 1*b* show a Watson Crick Wilkins helix seen in the direction of the helix axis, with one daughter nucleotide pair approaching (1*a*) and attached (1*b*). Figs. 1*c*, 1*d* show the same in side views.

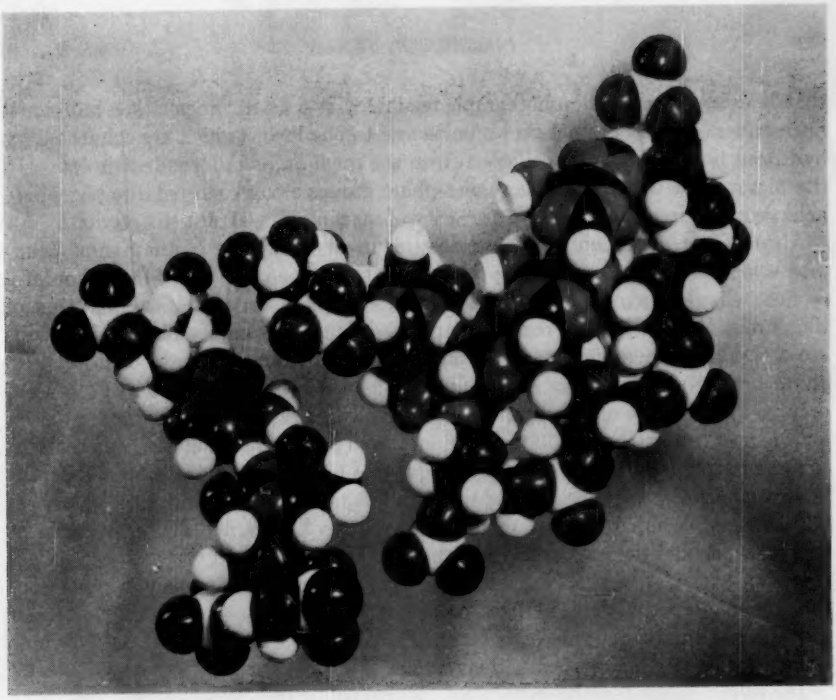

FIG. 1c

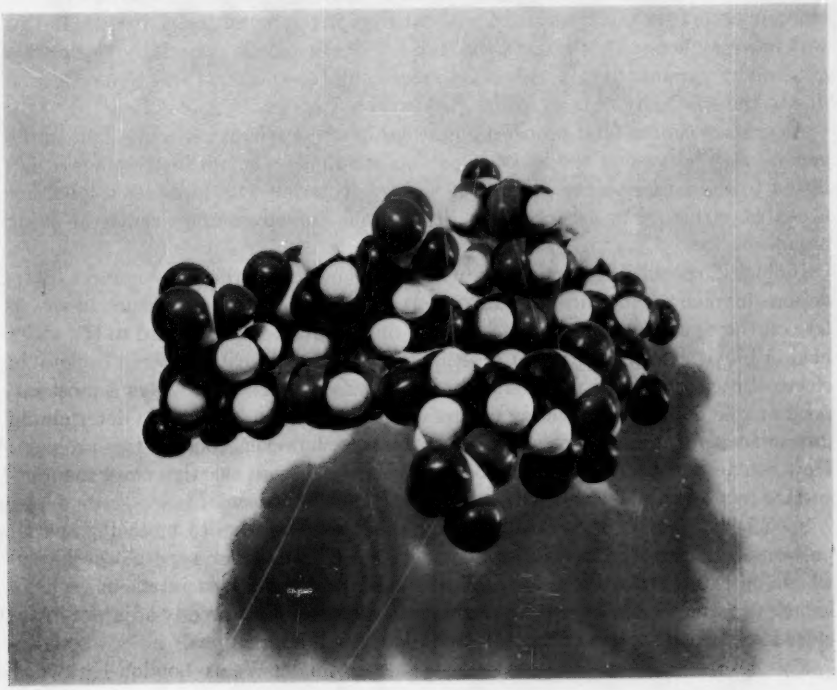

FIG. 1d

that is done to make the photograph readable; it does not mean that individual nucleotides, or pairs of nucleotides belonging to one layer (which are connected by hydrogen bonds only), are collected from the medium as independent units. (In the pictures we see one too many phosphate groups along each pentose phosphate helix section; this again is only for clarity of marking the latter helix sections.)

After proper collection of nucleotides (identical with those of the parent helix) into a mantle region surrounding the parent helix, the question of formation of a replica helix comes up. As the circumference of the mantle is larger than that of the parent DNA helix, the regularly arranged daughter pentose phosphate groups are occasionally separated by gaps from neighboring groups along the pentose phosphate helices of the replica. The formation of a replica DNA helix may then occur by the closure of some gaps, and finally all of them. That is facilitated by ionic concentration changes in the medium which permit the daughter helix sections to peel off from the parent helix. The sequence of base pairs of daughter and parent helices is the same. (Each base pair of the daughter helix might have the bases interchanged, compared with the arrangement in the parent helix.[6] The next generation should then again be the same as its grandparent.)

If the specificity of accurate selection of an adenine-thymine pair in preference to a guanine-cytosine pair is judged on the basis of the lower-limit estimates about expressions (2), it is not very high. It will still take considerable time to determine properly the actual magnitude of expressions (2).

The essential point in this replication mechanism is the selective collection, from the surrounding medium, of readily available constituent replica molecules for the construction of the daughter helix, without ripping the parent helix apart. In this way one may hope to understand the stability of the genetic material. There are a good many variants to this replication process, all based on this selective collection due to the specificity of the London-Kirkwood forces.

As the association (and proper orientation) between mirror molecules fails in the general case because of the effect of permanent dipole-moment interactions in addition to the polarizability interacting, a levo structure will duplicate a levo, not a dextro, structure in accordance with the behavior of macromolecules in living organisms.

Biological specificities occur in many other connections where the specific interactions involve nonidentical molecules. It would seem to be premature to speculate on the significance of the specificity of the London force in regard to the wider field of biological specificity. It is clear that complementarity, favored because of electrostatic, or because of general van der Waals stabilization,[7] plays a most important role not only in deciding macromolecular structure but also in determining intermolecular interactions. In several instances, however, there is this problem: How can a second macromolecule be formed which has an exactly complementary surface to a given particular surface of the first macromolecule?

Specificity because of complementarity is a specificity due to matching spatial patterns. In the following, attention is drawn to a complex type of specificity[8] which operates when a spatial pattern of different molecules interacts with an identical pattern next adjacent to it, so that corresponding directly adjacent molecules have specific van der Waals–London-Kirkwood interactions.

This interesting effect of high specificity due to van der Waals–London-Kirkwood

forces comes up in the following fashion. For simplicity of explanation, this effect can be illustrated in terms of two identical protein helices which possess occasional large side groups (Figs. 2*a* and 2*b*). The helices are alongside each other and also turned around their axes so that some of their corresponding side groups come to lie just between the helices next to each other. Thus the two proteins interlock, with corresponding side groups clinging together. The mutual orientation of these pairs of identical side groups is the energetically advantageous one (cf. Paper I, p. 343). Even if the polarizabilities are not extra-strong, the specificity

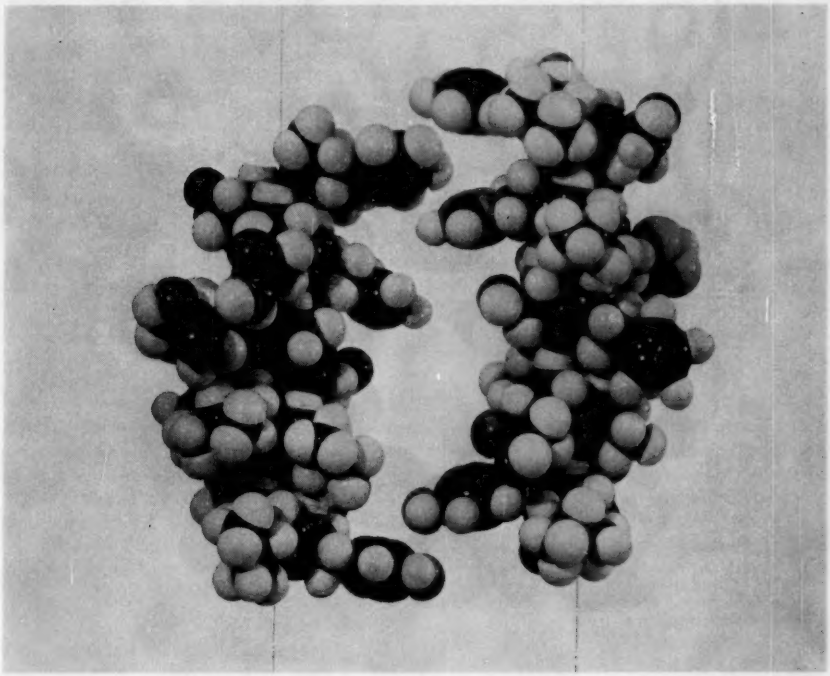

FIG. 2*a*

FIGS. 2*a*, 2*b*.—Specific interaction between two identical protein alpha helixes. Some corresponding side groups interlock and have proper mutual orientation. The sequence of specifically interacting pairs of side groups provides for a high degree of specificity.

becomes very high, owing to the matching pattern of pairwise identical side groups. Intervening spaces are expected to be filled, in a random fashion, by small molecules and ions from the medium. The interlocking of the two helixes may be considerably more intimate than shown in Fig. 2*b*.

To cite some further cases of interactions which favor associations of like molecules in preference to unlikes, and which may be due to the specificity of the London force, the phenomena connected with solubility may be mentioned.[9] The problem of differentiation,[10] in particular in the early development and growth of a fertilized egg, might also be related to rearrangement free energies of the type con-

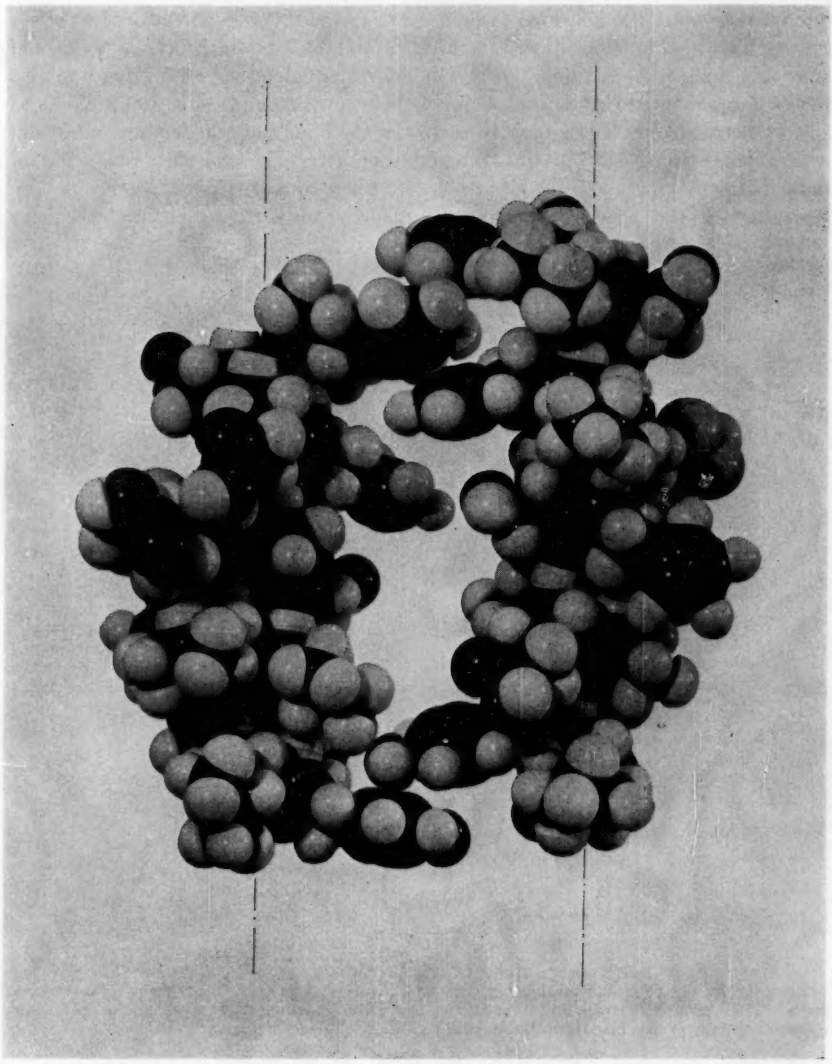

FIG. 2b

sidered here. The problem of specificity of growth-regulating substances, and the broader problem of antigen-antibody specificity, may be related to the model of protein specificity given above. And one should refer to the phenomenon of inverted synapsis,[4] mentioned in Paper I, which has led Muller[11] to consider the types of specificities discussed in the present notes. The degree of specificity of the van der Waals–London-Kirkwood forces is expected to be sufficient to account for this phenomenon.

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It may be adequate to use this more general title (rather than "Specificity of the London-Eisenschitz-Wang Force"); it refers to London force contributions of ground state and excited states, and it also refers to the Kirkwood-Shumaker force due to mobile protons. We avoid the word "resonance" because that term is usually used in a different connotation in chemical physics.

† On leave of absence at Gates, Crellin, and Church Laboratories, California Institute of Technology, 1956–1957. I should like to express my sincere thanks to Dr. Linus Pauling for the hospitality I enjoyed while there and for interesting discussions with him, Dr. Richard E. Marsh, and Dr. Herbert Segall. I should like to thank Dr. Pauling and Dr. Corey for the loan of molecule models, in particular of the alpha helix.

[1] Jerrold M. Yos, William L. Bade, and Herbert Jehle, these PROCEEDINGS, **43**, 341, 1957.

[2] The ordinates W_S I and W_S II in that figure are at least of the order of magnitude -0.1 to -1 if the interacting molecules are closest neighbors.

[3] John G. Kirkwood and John B. Shumaker, these PROCEEDINGS, **38**, 855 and 863, 1952.

[4] Jerrold M. Yos, William L. Bade, and Herbert Jehle, in *Symposium on Molecular Structure and Biological Specificity*, ed. L. Pauling and H. Itano (American Institute of Biological Sciences, 1957).

[5] H. A. Kramers and R. de L. Kronig, *Z. Physik*, **48**, 174, 1928.

[6] J. Herbert Taylor, P. S. Woods, and W. L. Hughes, these PROCEEDINGS, **43**, 122, 1957. Their findings might be related to this point; they might be due to any kind of specific London-Kirkwood association of pairs of identical DNA helixes.

[7] Linus Pauling, *Discussions Faraday Soc.*, **13**, 170, 1953; *Nature*, **161**, 707, 1948; F. Breinl and F. Haurowitz, *Z. physiol. Chem.*, **192**, 45, 1930; Stuart Mudd, *J. Immunol.*, **23**, 423, 1932; L. Pauling, D. H. Campbell, and D. Pressman, *Physiol. Rev.*, **23**, 203, 1943.

[8] A suggestion made by Dr. H. J. Muller a few years ago.

[9] H. C. Hamaker, *Physica*, **4**, 1058, 1937; H. R. Kruyt, *Colloid Science* (New York: Elsevier Publishers, Inc., 1952).

[10] Paul Weiss, in *Chemistry and Physiology of Growth*, ed. Parpartrun (Princeton, N. J.: Princeton University Press, 1949); in *Biological Specificity and Growth*, ed. Butler (Princeton, N. J.: Princeton University Press, 1955); *J. Embryol. Exptl. Morphol.*, **1**, Pt. 3, 181, 1953; *Hixon Symposium*, ed. Jeffress (New York: John Wiley & Sons, 1951); R. W. Sperry, *Growth Symposium*, **10**, 63, 1951; *Proc. First Intern. Neurochem. Symposium, Oxford*, **1954** (New York: Academic Press, Inc., 1955).

[11] H. J. Muller, *Am. Naturalist*, **56**, 32, 1922; *Proc. Roy. Soc. London, B*, **134**, 1, 1947; *Cold Spring Harbor Symposia Quant. Biol.*, **9**, 290, 1941.

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